# STUDIES ON EXPERIMENTALLY PRODUCED ANTIBODIES AGAINST PEROXYNITRITE MODIFIED-HISTONES

THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

## BIOCHEMISTRY

BY


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# CERTIFICATE

I certify that the work presented in the thesis entitled ***"Studies on experimentally produced antibodies against peroxynitrite modified–histones"*** has been carried out by ***Md. Asad Khan*** under my direct guidance and supervision, and is suitable for the award of ***Ph.D.*** degree in ***Biochemistry*** of the Aligarh Muslim University, Aligarh.

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*Dedicated*

*To*

*My Parents*

# Acknowledgements

*Since time immemorial, writing acknowledgement is customary and as a matter of fact a convention. Our accomplishment and achievements are to a one man's job. In science is generally done by a group of people I may put there;*
*"ART IS I; SCIENCE IS WE"*

*First of all I bow in reverence to Almighty, the most omnipotent, the most benevolent and merciful who provided me the zeal and zest combined with courage to accomplish my task.*

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*"Things turn out best for the people who make the best of the way things turn out"*

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Md. Asad Khan

**(Md. Asad Khan)**

**Contents** **Page Number**

# Abbreviations

| | | |
|---|---|---|
| $A_{276}$ | : | Absorbance at 260nm |
| $A_{280}$ | : | Absorbance at 280 nm |
| AP | : | Alkaline phospatase |
| APS | : | Ammonium persulphtae |
| A.U. | : | Arbitrary units |
| BSA | : | Bovine serum albumin |
| dsDNA | : | Double stranded DNA |
| CD | : | Circular dichroism |
| DTT | : | Dithiothreitol |
| DTPA | : | Diethylenetriamine pentacetic acid |
| EDTA | : | Ethylene diamine tetraacetic acid |
| ELISA | : | Enzyme linked immunosorbent assay |
| FT-IR | : | Fourier transformed-infra red |
| HPLC | : | High performance liquid chromatography |
| HSA | : | Human serum albumin |
| IgG | : | Immunoglobulin G |
| kDa | : | Kilo dalton |
| LDL | : | Low density lipoprotein |
| $MgCl_2$ | : | Magnasium chloride |
| µL | : | Microlitre |
| µM | : | Micromolar |
| M | : | Molar |
| nm | : | Nanometer |
| NO | : | Nitric oxide |
| $NO^{\bullet}$ | : | Nitric oxide radical |
| $NO_3$ | : | Nitrate |
| NOS | : | Nitric oxide synthase |
| 3-NT | : | 3-Nitrotyrosine |

| | | |
|---|---|---|
| nDNA | : | Native DNA |
| pDNA | : | Placental DNA |
| PON | : | Peroxynitrite |
| $ONOO^-$ | : | Peroxynitrite anion |
| PAGE | : | Polyacrylamide gel electrophoresis |
| PBS | : | Phosphate buffered saline |
| ROS | : | Reactive oxygen species |
| RNS | : | Reactive nitrogen species |
| rpm | : | Revolution per minute |
| RA | : | Rheumatoid arthritis |
| SDS | : | Sodium dodecyl sulphate |
| $O_2^{\cdot -}$ | : | Superoxide anion radical |
| SOD | : | Superoxide dismutase |
| SLE | : | Systemic lupus erythematosus |
| TEMED | : | N,N,N',N'-tetramethyl ethelenediamine |
| Tm | : | Melting temperature |
| Tris | : | Tris (hydroxymethyl) amino methane |
| Tm | : | Melting temperature |
| UV | : | Ultraviolet |
| V | : | Volt |

# Abstract

Reactive nitrogen and oxygen species are produced under physiological conditions. However, excess of these radicals may damage cellular lipids, proteins and nucleic acids. These reactive species have been implicated in many disease conditions including chronic inflammation, atherosclerosis, rheumatoid arthritis, some neurodegenerative diseases and systemic lupus erythematosus. Systemic lupus erythematosus and rheumatoid arthritis are autoimmune diseases with complex etiology and pathogenesis. The abnormal level of nitrotyrosine detected in tissues affected by above diseases have been attributed to peroxynitrite–mediated hypernitration of tyrosine residues in proteins. Peroxynitrite is a potent oxidant as well as nitrating agent and has *in vivo* existence. It is formed when nitric oxide reacts with superoxide radical. Peroxynitrite is a powerful pro-inflammatory substance and may increase vascular permeability in inflamed tissues.

In this doctoral thesis, physico-chemical and immunological studies have been carried out on histones modified by peroxynitrite with an objective of studying the possible role of oxidatively nitrated proteins in the initiation/progression of systemic lupus erythematosus and rheumatoid arthritis.

Analysis of UV absorption profile of peroxynitrite–modified H1, H2A, H2B and H3 histones revealed peak shift to higher wavelength and hyperchromicity at 276 nm compared to native histones. Furthermore, in case of modified–histones an additional peak was observed at 420 nm which corresponds to nitrotyrosine because a standard solution of 3-nitrotyrosine had given a similar peak under our experimental conditions. The hyperchromicity observed in peroxynitrite–modified histones' samples might be due to nitration of tyrosine residues which ultimately enhances the molar absorptivity compared to native histones. HPLC analysis confirmed generation of nitrotyrosine in peroxynitrite–modified histones. Fluorescence studies on native and peroxynitrite–modified histones showed significant decrease in the emission intensity of modified–histones. This quenching in emission intensity might be the consequence of introduction of nitro group in tyrosine residues.

The structural changes in peroxynitrite–modified histones were also studied by 1-anilinonaphthalene-8-sulfonic acid (ANS) – binding to native and modified–histones' samples. Upon modification by peroxynitrite the hydrophobic

clusters/patches in histones were exposed for ANS binding and it lead to increase in ANS fluorescence intensity and blue shift in emission wavelength. The observed blue shift could be the reflection of change in histones' structure from natively unfolded state to a partially folded form due to peroxynitrite–mediated nitration. Far–UV CD and FT–IR studies on native and peroxynitrite–modified histones concluded that the unfolded structure of histones converted into ordered structure after treatment with peroxynitrite. Melting studies demonstrated that interaction of histones with peroxynitrite caused increase in the thermal stability of peroxynitrite–modified histones compared to control. The stability of peroxynitrite–modified histones could be the consequence of formation of cross links and/or disulphide bridge(s). Furthermore, the appearance of ordered structure in modified–histones may also contribute to the observed rise in melting temperature. Results of polyacrylamide gel electrophoresis showed formation of high molecular weight species in peroxynitrite–treated histones. It may be linked to cross-linking and oligomerization of histones by the oxidative and nitrosative action of peroxynitrite.

Histones are conserved proteins and weak immunogen. However, they exhibit strong immunogenicity after acetylation and nitration. Rabbits challenged with peroxynitrite–modified H2A histone induced high titre antibodies; an indication that modification has generated powerful immunogenic epitopes. IgG antibodies purified from immune sera showed preferential binding with the immunogen as revealed by inhibition ELISA. The polyspecific nature of induced antibodies was established by its binding with peroxynitrite–modified forms of H2B, H1, H3 histones, HSA, proteins rich in tyrosine residues and nucleic acids. Mobility shift assay carried out in polyacrylamide gel reiterated the specific interaction between immunogen and induced antibodies.

Direct binding and inhibition ELISA experiments performed on serum samples of systemic lupus erythematosus and rheumatoid arthritis patients revealed that autoantibodies in the sera of these patients were recognizing peroxynitrite–modified H2A better than native H2A or native DNA. Normal human sera showed negligible binding with either antigen.

In conclusion, histones modified by laboratory synthesized peroxynitrite showed gross structural changes as revealed by physico–chemical results. Peroxynitrite–induced nitration and oxidation appears to have generated highly immunogenic epitopes on H2A histone. Experimentally produced antibodies against peroxynitrite–modified H2A were polyspecific because they showed specificity for the immunogen as well as cross-reaction with nitrated epitopes of other proteins. The preferential binding of peroxynitrite–modified histones by autoantibodies derived from systemic lupus erythematosus and rheumatoid arthritis sera points out the role of oxidatively modified and nitrated proteins in the initiation/progression of systemic lupus erythematosus and rheumatoid arthritis.

## List of Figures

## List of Figures

**List of Figures** **Page Number**

## List of Tables

# Introduction

Nucleosomes are the fundamental repeating units of eukaryotic chromatin. They package DNA into chromosomes inside the cell nucleus and control gene expression. Nucleosomes are made up of DNA and four pairs of histone proteins and resemble "beads on a string of DNA" when observed under electron microscope. H2A, H2B, H3 and H4 histones are part of the nucleosome while H1 histone is associated with linker DNA that joins two nucleosome particles.

## General information on histones

Histones are the chief proteins of chromatin and were discovered in the year 1884 by Albrecht Kossel. It has large proportion of positively charged amino acids, mainly arginine and lysine (9-30%). Core histones are highly conserved proteins and perform important role in the biology of the nucleus. Linker histone is less conserved than the core histones.

## Characteristics of histones

Histones derived from calf thymus are acid soluble, positively charged, small molecular weight proteins (11-23 kDa) found in association with DNA in chromatin of most eukaryotes. They lack tryptophan and with the exception of the arginine rich H3, they also lack cysteine or cystine. In higher animals and in many lower eukaryotes, there are five main types of histones. Two of them contain excess of arginine (H3 and H4) and the remaining three histones contain more lysine than arginine and are known as lysine rich histone fractions (H1, H2A and H2B). In some specialized cells, such as nucleated eukaryotes in birds & spermatozoa of various animals, additional histone fractions have been found which differ in amino acid composition from the five main histone species (Hnilica *et al.*, 1972; Delange and Smith, 1971; Stellwagen and Cole, 1969; Wigle and Dixon, 1971).

Histones act as spools around which DNA is wrapped and they play critical role in gene regulation. This enables the compaction which is necessary to fit the large genomes of eukaryotes inside cell nuclei. The compacted molecule is 50,000 times shorter than an unpacked molecule. Two each of the H2A, H2B, H3 and H4 assemble to form one octameric nucleosome core particle by wrapping 146 base pairs of DNA around the protein spool. The linker histone (H1) binds the nucleosome and thus locking the DNA into place and allowing the formation of

higher order structure. This involves the wrapping of DNA around nucleosomes with approximately 50 base pairs of DNA spaced between each nucleosome.

Fraction nomenclature of the five histones in calf thymus is as follows;

| Histone type | Bradbury nomenclature | Johns nomenclature | Molecular weight (kDa) |
|---|---|---|---|
| Lysine rich | H1 | KAP or F1 or 1 | 21.50 |
| Slightly Lysine rich | H2A | Lak or ALK or F2a2 or IIb1 | 14.00 |
| Slightly Lysine rich | H2B | KAS or KSA or F2b or IIb2 | 13.77 |
| Arginine rich | H3 | ARE or ARK or F3 or III | 15.32 |
| Arginine rich | H4 | GRKor F2aI or IV | 11.28 |

Source: Elgin and Weintraub, 1975

Structure of nucleosome core is consist of two H2A-H2B dimers and two H3-H4 dimers. The 'core' histones (H2A, H2B, H3 and H4) are relatively similar in structure and are highly conserved. They also share the feature of long 'tails' on one end of the amino acid structure—this being the location of post-transcriptional modification. In all, histones make five types of interactions with DNA;

i. Helix-dipoles from alpha-helices in H2B, H3 and H4 causes a net positive charge to accumulate at the point of interaction with negatively charged phosphate groups on DNA.

ii. Hydrogen bonds between the DNA backbone and the amino group on the main chain of histone proteins.

iii. Non-polar interactions between the histone and deoxyribose sugars on DNA.

iv. Salt links and hydrogen bonds between side chains of basic amino acids (especially lysine and arginine) and phosphate oxygen on DNA.

v. Non-specific minor groove insertions of the H3 and H2B N-terminal tails into two minor grooves each on the DNA molecule.

The highly basic natures of histones, aside from facilitating DNA-histone interactions, contribute to the water solubility of histones. Histones are subject to post-translational modification by enzymes primarily on their N-terminal tails.

Such modifications include methylation, citrullination, acetylation, phosphorylation, ubiquitination and ADP-ribosylation. This affects their function of gene regulation. Post-translational modifications of histones alter their interaction with DNA and other nuclear proteins.

**Immunogenicity of histones**

Antibodies directed against defined regions of histone represent one of the most specific probes for studying the surface topography of nucleosome and chromatin and for monitoring post-translational modifications. In recent past, about two dozen antigenic determinants/epitopes of the four core histones have been identified. Most of these epitopes, known as continuous epitopes, correspond to linear stretches of 6-20 amino acid residues. It is noteworthy that all terminal regions of the four histones, except carboxy terminal residues of H4, correspond to continuous epitopes. Antibodies directed against the epitopes can be used to determine which regions of histone molecules are exposed at the surface of nucleosome and are useful probes for analyzing chromatin structure and function.

Size of macromolecules play important role in its immunogenecity. The best immunogens tend to have a molecular mass approaching 10 kDa. Generally, substances with a molecular mass of less than 5-10 kDa are poor immunogens. Furthermore, size alone is not sufficient for immunogenicity, other properties are also needed. All four levels of protein organization—primary, secondary, tertiary and quaternary contribute to the structural complexity of a protein and hence affect its immunogenicity (Goldsby *et al.*, 1999).

Native core histones are poor immunogens. Their weak immunogenecity has been ascribed to highly conserved sequences and presence of few aromatic amino acid residues (Stoller, 1978; Bustin, 1979). Several laboratories have successfully prepared antibodies to nucleosome (Mura *et al.*, 1980), but reports are lacking on production of antibodies to native core histones. However, animals immunized with core histones complexed with RNA or DNA induced good antibody response in experimental animals (Stoller and Ward, 1970). Similarly, triacetylated histones complexed with RNA have been reported to be immunogenic (Muller *et al.*, 1991). Use of anti-histone antibodies as a probe has allowed detection of many details on the surface of the chromatin subunits

(Muller *et al.*, 1982a; Muller *et al.*, 1982b). Anti-histone antibodies as immunochemical reagents have also been useful in the elucidation of the role of histone post-translational modification in transcription and replication (Hebbes *et al.*, 1988; Turner, 1991; Perry *et al.*, 1993).

**Reactive nitrogen species**

Free radicals are reactive molecules with unpaired electrons. They represent an important arm of host defense against a variety of pathogens (Zahrt and Deretic, 2002). Reactive nitrogen intermediates (as well as oxygen) are directly toxic to invading pathogens. These reactive species also activate redox-sensitive signaling pathways, such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), that in turn regulates the transcription of proinflammatory proteins such as cytokines (Rahman *et al.*, 2006). During inflammation, overproduction of free radicals may lead to a break in immune tolerance, increased tissue damage and altered enzyme function. Reactive oxygen and nitrogen intermediates (RONI) play an important role in cellular signaling processes when produced at low levels. At higher levels, these molecules can cause direct toxicity to cells and induce modifications in lipids, amino acids and DNA (Oates and Gilkeson, 2006). However, to minimize damage by RONI, both enzymatic and non-enzymatic antioxidant defense plays important role.

*(i) Nitric oxide*

Nitric oxide ($NO^{\bullet}$) is probably the most relevant free radicals having broad spectrum of physiological and pathophysiological function. They are readily converted by enzymes or nonenzymic chemical reactions into reactive non-radical species, which can in turn give rise to new radicals. Radical and non-radical reactive nitrogen species include $NO^{\bullet}$, nitrogen dioxide ($^{\bullet}NO_2$), nitrous acid ($HNO_2$), nitrosyl cation ($NO^{\bullet}$), nitrosyl anion ($NO^{-}$), dinitrogen tetroxide ($N_2O_4$), dinitrogen trioxide ($N_2O_3$), peroxynitrite ($ONOO^{-}$), peroxynitrous acid (ONOOH), alkyl peroxynitrites and nitronium cation ($NO_2^{+}$).

Nitric oxide is produced from L-arginine by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS: neuronal NOS (nNOS or NOS-I) originally identified in brain, inducible NOS (iNOS or NOS-II) originally identified in macrophages, and endothelial NOS (eNOS or NOS-III) originally

identified in endothelial cells (Ghafourifar *et al.*, 2001). iNOS is expressed in macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells and cardiac myocytes in response to inflammatory cytokines, The expression of iNOS is regulated both at the level of transcription and iNOS mRNA stability. Catalytic activity of iNOS is regulated by the availability of the substrate, L-arginine, and of the cofactors, NADPH and tetrahydrobiopterin. Induction of iNOS expression is complemented by co-induction of cationic amino acid transporter proteins (increase in the intracellular L-arginine level) and GTP cyclohydrolase (key enzyme of tetrahydrobiopterin synthesis). Increased $NO^{\bullet}$ production via induction of iNOS has been suggested as a major mechanism by which cytokines mediate inflammatory responses and development of autoimmune diseases. (Turko and Murad, 2002).

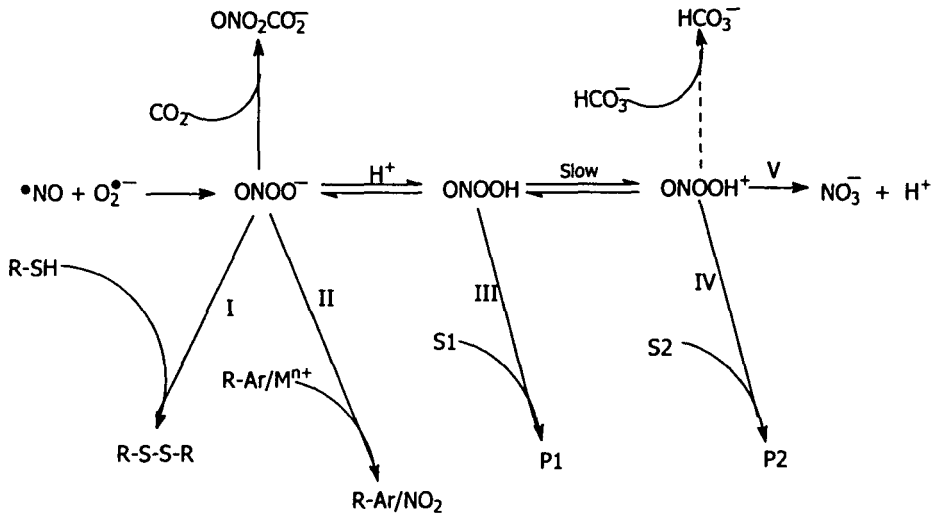
*(ii) Peroxynitrite*

Peroxynitrite is powerful oxidant and nitrating agent. It is formed by reaction of superoxide (a reactive oxygen species) and nitric oxide (a reactive nitrogen species). Peroxynitrite has been reported in endothelial cells (Palmer *et al.*, 1988), macrophages (Ischiropoulos *et al.*, 1992a), neuronal cells (Lafon-Cazal *et al.*, 1993), neutrophils (Carreras *et al.*, 1994) and smooth muscle cells (Schulz *et al.*, 1991) etc.

The decay of peroxynitrite is strongly influenced by temperature and transition metal ions which catalyze isomerisation of peroxynitrite. In aqueous alkaline solutions, peroxynitrite anion is stable for weeks if kept frozen in dark (Gmelin, 1996). The peroxynitrite anion shows maximum absorption at 302 nm with an extinction coefficient of 1670 $M^{-1}cm^{-1}$ (Hughes and Nicklin, 1968). Contrary to above, peroxynitrous acid gives maximum absorption at 345 nm with an extinction coefficient of 80 $M^{-1}cm^{-1}$; shoulder at 250 nm with an extinction coefficient of 600 $M^{-1}cm^{-1}$ has also been observed.

Peroxynitrite has a biological half life of 10–20 milliseconds and possess the ability to destroy critical cellular components (Beckman *et al.*, 1990; Denicola *et al.*, 1998). It can oxidize thiols (Radi *et al.*, 1991b) and/or nitrate DNA (Inoue and Kawanishi, 1995; Salgo *et al.*, 1995), proteins (Ischiropoulos and Al-Mehdi, 1995; Grune *et al.*, 2001) and lipids (Radi *et al.*, 1991a). At

physiological pH, about 20% of peroxynitrite is protonated to peroxynitrous acid (HOONO) which has a pka of 6.8. This fraction of peroxynitrous acid play significant role in the biological activity. For example, one- and two-electron oxidations can be performed by peroxynitrite. Different mechanisms of reactivity of peroxynitrite have been proposed (Denicola *et al.*, 1996) and the same has been shown in scheme 1. As depicted in reaction I peroxynitrite anion ($ONOO^-$) may directly react with sulfhydryls (RSH) to yield corresponding disulfide (RSSR). Peroxynitrite anion may also nitrate aromatic rings (R-Ar) to yield nitro–derivatives (reaction II). When protonated to peroxynitrous acid (ONOOH), it can react with biomolecules (S1) (reaction III). It can also undergo a rate-limiting transition to vibrationally activated intermediate (ONOOH*). The activated intermediate can isomerize to nitric acid (reaction V) or oxidize a target molecule (S2; such as benzoate or DMSO) with a reactivity similar to that of hydroxyl radical (reaction IV)




**Scheme 1.** Reactions of peroxynitrite

Furthermore, the peroxynitrite anion may also react with carbon dioxide (physiological concentration of the latter is about 1 mM in blood plasma) with a rate constant of 3 x $10^4$ $M^{-1}s^{-1}$ at 25°C (Lymar and Hurst, 1995). This reaction produces 1–carboxylato–2–nitrosodioxidane ($ONOOCO_2^-$) which is mainly responsible for toxicity of peroxynitrite. In the absence of other reactants, $ONOOCO_2^-$ undergoes homolysis and forms nitrogen dioxide and trioxocarbonate

radicals with a yield of 4–30% (Coddington *et al.*, 1999; Bonini *et al.*, 1999; Meli *et al.*, 2002; Lymar and Hurst, 1998) which isomerises to nitrate and carbon dioxide.

Since its published discovery in the year 1987, peroxynitrite has received overwhelming attention of biomedical researchers because of its involvement in the regulation of blood pressure and activities in brain, lungs, kidneys, stomach, gut etc.

Numerous studies have demonstrated that peroxynitrite toxicity is due to its ability **(a)** to oxidize thiols and thiol containing proteins and membrane lipids (Radi *et al.*, 1991a; Radi *et al.*, 1991b) and **(b)** to nitrate phenols including free and protein bound tyrosines. Proteins react with peroxynitrite to form a stable yellow protein-bound adduct identified as nitrotyrosine (Ischiropoulos *et al.*, 1992b).

Because of its oxidant nature, peroxynitrite can directly react with electron-rich groups; such as sulfhydryls (Radi *et al.*, 1991b), iron-sulfur centers (Castro *et al.*, 1994), zinc-thiolates (Crow *et al.*, 1995) and active site sulfhydryl in tyrosine phosphatases (Takakura *et al.*, 1999). Curiously, the reaction rate constant for these second-order reactions varies from $10^3$ to $10^6$ $M^{-1}$ $s^{-1}$. Although peroxynitrite is a strong oxidant, but its anion ($ONOO^-$) reacts directly with molecules carrying partial positive charges. In addition to nitrotyrosines, peroxynitrite can also produce novel products such as nitrotryptophan and nitrated lipids that may emerge as important biological markers *in vivo*.

**Peroxynitrite as an oxidant and nitrating agent**

Oxidation reactions of peroxynitrite include DNA damage, leading to base modification (Douki *et al.*, 1996) and mutations as well as single– and double–strand breaks (Salgo *et al.*, 1995; Szaboè and Ohshima, 1997). Peroxynitrite also causes one- or two-electron oxidation of sulfhydryls (Quijanoet *et al.*, 1997) leading to thiyl radical formation, radical chain reactions and depletion of thiol pools. Nitration reactions are predominantly nitration of phenols, such as nitration of tyrosine residues in proteins. Protein tyrosine nitration by peroxynitrite may interfere with phosphorylation/dephosphorylation signaling pathways (Kong *et al.*, 1996; Li *et al.*, 1998). Because of its cytotoxic action on bacteria (Zhu *et al.*, 1992) or other invading pathogens, the peroxynitrite may prove useful. Inflammatory cells (macrophages and neutrophils) produce large amount of both

nitric oxide and superoxide which rapidly combines to form peroxynitrite (Huie and Padmaja, 1993; Kissner *et al.*, 1997; Ischiropoulos *et al.*, 1992a). However, excessive production of peroxynitrite can damage normal tissues. Indeed, the formation of protein 3-nitrotyrosine *in vivo* has been shown in a number of inflammatory conditions in humans and experimental animals (Ischiropoulos, 1998).

**Scavengers of peroxynitrite**

Peroxynitrite and peroxynitrite–derived radicals may be neutralized by several molecules. In animal models of inflammation and reperfusion injury both uric acid and ascorbic acid have shown beneficial effects (Szabó *et al.*, 2007; Yeo *et al.*, 2008). Scavenger action of these acids is more pronounced on peroxynitrite derived radicals. Simple thiol-based antioxidants, such as mercaptoalkylguanidines, N-acetylcysteine, cystein and dihydrolipoic acid have shown reduction in peroxynitrite-mediated toxicity and reduce 3-nitrotyrosine immunoreactivity in various pathophysiological conditions (Szabó *et al.*, 2007). Ebselen and several unsaturated oils have exerted protective effects in numerous models of inflammation and reperfusion injury (Sugiura *et al.* 1999; Daiber *et al.*, 2000; Noiri *et al.*, 2001). Some of these actions are likely to be associated with the ability of the compound to neutralize peroxynitrite. Indeed, the selenium-containing glutathione peroxidase and other enzymes such as catalase, superoxide dismutase have been shown to readily reduce peroxynitrite to nitrite *in vitro*, but their protective role in *in vivo* has not been confirmed (Afonso *et al.*, 2007). Several marketed antioxidants (such as dithiothreitol and DMSO) have been demonstrate to scavange peroxynitrite derived secondary radicals. Whether the antioxidant effects of these compounds will contribute to their therapeutic actions remain to be seen. Desferrioxamine has been typically used *in vivo* as an iron chelator and therefore an inhibitor of iron dependent generation of hydroxyl radicals. However, desferrioxamine has also inhibited peroxynitrite-dependent oxidation and nitration by reacting with the $CO_3^{-\bullet}$ and $NO_2^{\bullet}$ radicals (Bartesaghi *et al.*, 2004). Recent studies demonstrate that tyrosine-containing peptides can also act as scavengers of peroxynitrite derived radicals at the cellular level and may

neutralize the cytotoxic effects of both exogenous and endogenous peroxynitrite, inhibiting its pro-apoptotic effect on neurons (Ye *et al.*, 2007).

**Peroxynitrite–induced cytotoxicity**

Under physiological conditions, production of peroxynitrite remains low and oxidative damage is neutralized by endogenous antioxidant defenses (Radi *et al.*, 2002a; Radi *et al.*, 2002b; Trujillo *et al.*, 2008). Even modest increase in the simultaneous production of superoxide and nitric oxide will greatly stimulate peroxynitrite formation, a 10-fold increase in superoxide and nitric oxide will causes 100-fold increase in peroxynitrite formation. The generation of a moderate flux of peroxynitrite over a long period of time will result in substantial oxidation and potential destruction of host cellular constituents, leading to the dysfunction of critical cellular processes, disruption of cell signaling pathways and the induction of cell death through both apoptosis and necrosis (Virag *et al.*, 2003). Hence, the production of peroxynitrite can be instrumental in the development of many pathological processes *in vivo*.

Although not a free radical, peroxynitrite is much more reactive than either nitric oxide or superoxide (Beckman, 1990; Beckman and Koppenol, 1996). The half-life of peroxynitrite is short (10–20 milli seconds) but sufficient to cross biological membranes, diffuse one to two cell diameters (Denicola *et al.*, 1998) and allow significant interactions with most critical biomolecules (Pryor and Squadrito, 1995). Kinetic studies indicated that peroxynitrite oxidizes target molecules through two distinct mechanisms. First, peroxynitrite and its protonated form (peroxynitrous acid; ONOOH) can cause direct oxidative modifications through one- or two-electron oxidation processes (Beckman *et al.*, 1996). Second, peroxynitrite can yield hydroxyl radical and nitrogen dioxide radical ($NO_2^{\bullet}$) during the homolytic decomposition of peroxynitrous acid. However, the formation of hydroxyl radical by this mechanism probably plays only a minor role in *in vivo* (Radi, 1998) because of the particularly rapid reaction of peroxynitrite with carbon dioxide.

**Peroxynitrite in apoptosis and necrosis**

Mitochondria are involved in energy production, calcium homeostasis, control of various biosynthetic pathways etc. Disruptions of mitochondrial

functions have been implicated in diabetes, atherosclerosis, ischemic heart diseases, stroke, aging, neurodegenerative diseases etc. The mitochondrial toxicity of peroxynitrite results both from direct oxidative reactions as well as from free radical-mediated damage (Radi *et al.*, 2002a; Radi *et al.*, 2002b). The damaging potential of peroxynitrite is due to direct oxidation as well as radical-mediated nitration reactions. These properties allow peroxynitrite to significantly alter function of a number of proteins, to degrade membrane structure by peroxidizing lipids, to turn off crucial metabolic functions within mitochondria and to inflict serious damage to nucleic acids. Once the level of cellular damage inflicted by peroxynitrite supercedes any possibility of repair, the cell eventually dies via one of the two main pathways of cell demise, necrosis or apoptosis. Necrosis is associated with loss of cellular ATP, leading to membrane disruption, release of noxious cellular debris, and the development of secondary inflammation. In contrast, apoptosis occurs in a well-choreographed sequence of morphological events characterized by nuclear and cytoplasmic condensation with blebbing of the plasma membrane. Apoptosis is orchestrated by the proteolytic activation of cysteine proteases known as caspases, that requires preserved ATP levels to proceed properly and which may be triggered either by the activation of death receptors (extrinsic pathway) or by the permeabilization of the outer membrane of mitochondria (intrinsic pathway) (Bouchier-Hayes *et al.*, 2005; Newmeyer and Ferguson-Miller, 2003). Peroxynitrite has been associated with apoptosis in HLA-60 cells (Lin *et al.*, 1995), PC12 cells (Estevez *et al.*, 1995), fibroblasts (Raghuram *et al.*, 1999), SN 4741 dopaminergic neurons (Shacka *et al.*, 2006), SH-SY5Y neuroblastoma cells (Saeki *et al.*, 2002), primary neurons (Bonfoco *et al.*, 1995; Estevez *et al.*, 1998; Kanski *et al.*, 2005; Kanski *et al.*, 2005), astrocytes (Zhu *et al.*, 2006) and oligodendrocytes (Zhang *et al.*, 2006) endothelial cells (Dickhout *et al.*, 2005; Walford *et al.*, 2004), beta islet cells (Delaney *et al.*, 1996; Suarez-Pinzon *et al.*, 1997), neutrophils (Fortenberry *et al.*, 1998; Taylor *et al.*, 2004), chondrocytes (Whiteman *et al.*, 2004), cardiomyocytes (Arstall *et al.*, 1999; Levrand *et al.*, 2006) and renal tubular cells (Allen *et al.*, 2003). Several mechanisms have been proposed to explain the activation of the apoptotic program by peroxynitrite. However, a common pathway involving

the mitochondria and permeabilization of their outer membrane is emerging as a key feature of peroxynitrite-mediated apoptosis which is a prominent feature of peroxynitrite-mediated cell death.

Whereas apoptosis is a typical consequence of low to moderate concentrations of peroxynitrite, exposure of cells to higher concentrations of the oxidant has been associated with necrosis (Bonfoco *et al.,* 1995; Virag *et al.,* 2003). Peroxynitrite-dependent cell necrosis is not a purely passive phenomenon, but instead is mediated by a complex process involving DNA damage and activation of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP-1) ( Szabo *et al.,* 1996). Poly (ADP-ribose) polymerase is a member of the PARP enzyme family consisting of PARP-1 and many additional poly (ADP-ribosylating) enzymes. An important function of PARP-1 is to allow DNA repair and cell recovery in conditions associated with a low degree of DNA damage as a result, the loss of $NAD^+$ leads to a marked decrease in the cellular pools of ATP, resulting in cellular dysfunction and cell death via the necrotic pathway (Ha and Snyder, 1999; Liaudet and Oddo, 2003). As such, PARP cleavage has been proposed to function as a molecular switch between apoptotic and necrotic modes of cell death (Boulares *et al.,* 1999; Levrand *et al.,* 2006; Los *et al.,* 2002).

**Reaction of peroxynitrite with proteins**

*(i)* *Reactions with proteins containing transition metal:* The direct reaction of peroxynitrite with transition metal centers is among the fastest known for peroxynitrite (Alvarez and Radi, 2003). Peroxynitrite thus can modify proteins containing a heme prosthetic group; such as hemoglobin (Boccini and Herold, 2004), myoglobin (Herold *et al.,* 2003), or cytochrome C (Thomson *et al.,* 1995) by oxidizing ferrous heme into the corresponding ferric form. Similarly, peroxynitrite can inactivate inducible nitric oxide synthase (iNOS) by oxidative modification of its heme group (Huhmer *et al.,* 1997), a reaction which might serve as a feedback negative regulation of peroxynitrite generation under inflammatory conditions. Peroxynitrite can also react with iron-sulfur clusters thereby inactivating mitochondrial aconitase (Castro *et al.,* 1994) and phoshogluconate dehydratase (Keyer and Imlay, 1997) involved in critical metabolic processes. Similarly, reaction of peroxynitrite with $Zn^{2+}$ sulfur motifs of

endothelial nitric oxide synthase (eNOS) (Zou *et al.*, 2004) and alcohol dehydrogenase (Crow *et al.*, 1995) causes inactivation of these enzymes.

*(ii)* *Reactions with cysteine:* Reaction of peroxynitrite with various amino acids alters protein structure and function. Upon reaction with cysteine, peroxinitrite causes thiol oxidation (Radi *et al.*, 1991b). Oxidation of critical cysteine residues inactivates many enzymes of cellular energetic processes; some examples are; glyceraldehyde-3-phosphate dehydrogenase (Buchczyk *et al.*, 2003; Souza and Radi, 1998), creatine kinase (Konorev *et al.*, 1998), complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase) as well as complex V (ATP synthase) of the mitochondrial respiratory chain (Radi *et al.*, 1994, Radi *et al.*, 2002a; Radi *et al.*, 2002b). Furthermore, these enzymes are also inactivated by nitration of tyrosine residues. The susceptibility of cells to peroxynitrite toxicity largely depends on the amount of intracellular glutathione. Depletion of glutathione enhances peroxynitrite toxicity and tissue injury during circulatory shock (Cuzzocrea *et al.*, 1998; Cuzzocrea *et al.*, 1999). A correlation between glutathione depletion and enhanced peroxynitrite toxicity has also been respected in some neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis (Marshall *et al.*, 1999; Vargas *et al.*, 2006).

*(iii)* *Reaction with tryptophan, methionine and histidine:* Peroxynitrite mediated oxidation of methionine generates methionine sulfoxide and to a lesser extent ethylene and dimethyldisulfide (Alvarez and Radi, 2003). Methionine oxidation can also inhibit alpha-1-antiproteinase which then loses ability to inactivate proteases most notably elastase (Whiteman *et al.*, 1996). Peroxynitrite can also oxidize tryptophan (Alvarez and Radi, 2003) to *N*-formylkynurenine, oxindole, hydropyrroloindole and nitrotryptophan, but relevance of these products is yet to be understood (Alvarez and Radi, 2003; Yamakura and Ikeda, 2006). Peroxynitrite modifies histidine through a radical mechanism, forming a histidinyl radical, a mechanism involved in the inactivation of Cu–Zn superoxide dismutase by peroxynitrite (Alvarez *et al.*, 2004; Yamakura and Ikeda, 2006; Yamakura *et al.*, 2005).

*(iv)* *Reaction with tyrosine:* Tyrosine nitration by peroxynitrite is a covalent modification that results in addition of a nitro ($-NO_2$) group adjacent to the

hydroxyl group on the aromatic ring of tyrosine residues (Gow *et al.*, 2004). It may cause generation of new epitopes, changes in enzymes catalytic activity, altered cytoskeletal organization and impaired cell signal transduction (Virag and Szabo, 2000). Tyrosine nitration occurs through radical mechanism in which a hydrogen atom is first abstracted from tyrosine to form a tyrosyl radical that quickly combines with nitrogen dioxide radical to produce 3-nitrotyrosine. Nitrotyrosine performs a secondary reaction where it combines with another tyrosyl radical to form dityrosine (Beckman, 1996; Beckman *et al.*, 1996; Ischiropoulos, 2003; Radi, 2004). Although the nitrogen dioxide radicals involved in the reaction may come from homolysis of peroxynitrite to form hydroxyl and nitrogen dioxide radicals (like $HO^{\bullet}$ and $NO_2^{\bullet}$), they most likely come from reaction of peroxynitrite with $CO_2$ producing $CO_3^{-\bullet}$ and $NO_2^{\bullet}$ radicals (Alvarez and Radi, 2003; Radi, 2004; Turko and Murad, 2002). A second mechanism of tyrosine nitration relies on the generation of the nitrogen dioxide radical from various heme-peroxidases (mainly myeloperoxidase and eosinophil peroxidase) in presence of hydrogen peroxide (Gaut *et al.*, 2001; Van der Vliet *et al.*, 1997; Wu *et al.*, 1999). This may serve as a recycling mechanism to reuse products formed from superoxide and $NO^{\bullet}$. Therefore, nitrotyrosine formation technically must be considered a marker of "nitrosative" stress.

The advent of proteomic analyses has confirmed that nitration is a highly selective process, limited to specific tyrosine residues on a surprisingly small number of proteins (Aulak *et al.*, 2001; Gow *et al.*, 2004; Kanski *et al.*, 2005a; Kanski *et al.*, 2005b). Nitration on specific tyrosine residues is promoted by the exposure of the aromatic ring to the surface of the protein, the location of the tyrosine on a loop structure, its association with a neighboring negative charge and absence of proximal cysteines (Souza *et al.*, 1999). Tyrosine nitration may also be favored in hydrophobic environment considering that peroxynitrous acid can readily pass through lipid membranes (Bartesaghi *et al.*, 2006; Turko and Murad, 2002).

**Protein oxidation**

The reaction of intracellular peroxynitrite with cellular structures will lead to a multitude of damages including protein modification. Peroxynitrite–induced

modification of proteins includes oxidation (of methionine, cysteine, tryptophane or tyrosine residues) and nitration (of tyrosine and/or tryptophane residues). However, the product of protein–peroxynitrite interaction investigated most often is 3-nitrotyrosine (Beckman and Koppenol, 1996; Kong *et al.*, 1996; Ischiropoulos *et al.*, 1992b). Enzymes containing a redox active transition metal center are the prime targets (Beckman and Koppenol, 1996). Reactions of peroxynitrite are affected by the local pH and the microenvironment with hydrophobic membrane compartments favoring nitration and aqueous environments favoring oxidation. Moreover, carbon dioxide reacts with peroxynitrite resulting in the formation of nitroso–peroxocarbonates (Radi *et al.*, 2001). The ubiquitous presence of $CO_2$ at high concentration may favor this reaction route. As nitroso-peroxo carbonates divert peroxynitrite induced protein modifications toward nitration, $CO_2$ is now considered as key determinant of peroxynitrite chemistry. Peroxynitrite can directly oxidize prosthetic groups of a protein, e.g. hemoglobin, or directly react with the peptide chain leading to conformational and functional changes with severe biological consequences. Enzymes with critical cysteine residues can be inactivated by peroxynitrite (Takakura *et al.*, 1999). Finally, oxidation of a critical cysteine residue was found to activate matrix metalloproteinases where the cysteine residue is in the auto-inhibitory domain of the proenzyme (Okamoto *et al.*, 1997). In case of peroxiredoxine (thiol-dependent peroxidases) oxidation of cysteine residues to disulfide (via sulfenic acid) is part of the catalytic cycle (Bryk *et al.*, 2000). Protease inhibitory properties of α1-antiproteinase are lost after peroxynitrite-mediated oxidation of its critical methionine residues to methionine sulfoxide (Moreno and Pryor, 1992). The oxidation of methionine is readily reversed by methionine sulfoxide reductase at the expenses of thioredoxin.

**Protein carbonyl**

Generation of free radicals and oxidants is common during normal metabolism (Tobias *et al.*, 2007). It may also be a sign of normal aging or a hallmark of pathologic processes (Klaunig and Kamendulis, 2004; Emerit *et al.*, 2004; Bailey, 2003; Fubini and Hubbard, 2003). It is true both for reactive oxygen– and reactive nitrogen species. The peroxynitrite anion ($ONOO^-$) is a highly reactive molecule (Gow *et al.*, 1996; Stadtman, 2001; Castegna *et al.*, 2003;

Souza *et al.*, 1999) and has low diffusion distance in biological tissue (Ischiropoulos and Al-Mehdi, 1995). The most prominent analyzing marker for protein oxidation is carbonyl contents of proteins (Berlett and Stadman, 1997; Chevion *et al.*, 2000; Shacter, 2000; Beal, 2002). Carbonyl appears (C=O) as a consequence of oxidative modification of the side chains of lysine, proline, arginine and threonine (Berlett *et al.*, 1998). These moieties are chemically stable. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins by either the α-oxidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative (Berlett and Stadman, 1997). Determination of protein C=O groups as biomarker of oxidative stress has some advantages over measurement of other oxidation products because of stability of former. Accumulation of protein carbonyls has been observed in Alzheimer's disease, diabetes (Ceriello, 2008), inflammatory bowel disease, systemic lupus erythematosus, rheumatoid diseases, sepsis, chronic renal failure, respiratory distress syndrome (Dalle-Dome *et al.*, 2003; Ghafourifar *et al.*, 2008), cancer (Yilmaz *et al.*, 2003) etc.

**Reaction of of peroxynitrite with lipids**

A major aspect of peroxynitrite-dependent cytotoxicity relies on its ability to trigger lipid peroxidation in membranes, liposomes and lipoproteins by abstracting a hydrogen atom from polyunsatturated fatty acids (Radi *et al.*, 1991a). Resulting products include lipid hydroperoxyradicals, conjugated dienes, and aldehydes (Denicola and Radi, 2005). Such radicals in turn attack neighboring polyunsatturated fatty acids, generating additional radicals which propagate free radical reactions and ultimately degeneration of membrane lipids (Hogg and Kalyanaraman, 1999; Radi *et al.*, 1991a) causing changes in membrane permeability and fluidity (Smith *et al.*, 1999). Peroxynitrite may play a critical role in inflammatory diseases of the nervous system by initiating peroxidation of myelin lipids leading to demyelination (Shi *et al.*, 1999; Smith *et al.*, 1999; Van der Veen and Roberts, 1999). It can also act as a potent oxidizing agent towards low-density lipoprotein (LDL) (Leeuwenburgh *et al.*, 1997; Trostchansky *et al.*, 2003). Peroxynitrite–modified LDL binds to scavenger receptors with high

affinity leading to the accumulation of oxidized cholesteryl esters and foam cells which are key molecules of atherogenesis (Graham *et al.*, 1993; Guy *et al.*, 2001; Hogg *et al.*, 1993). Finally, the interaction of peroxynitrite with membrane lipids may lead to various nitrated lipids which can act as potential biological mediators of signal transduction under physiological and pathological conditions (Baker *et al.*, 2004).

**Reactions of peroxynitrite with DNA**

Peroxynitrite can damage DNA by introducing oxidative modifications in both nucleobases and sugar-phosphate backbone. Among the four nucleobases, guanine is the most reactive with peroxynitrite due to its low reduction potential (Yu *et al.*, 2005). The major product of guanine oxidation is 8-oxoguanine, which upon further reaction with peroxynitrite yields cyanuric acid and oxazolone (Niles *et al.*, 2006). Ultimately, guanine oxidation by peroxynitrite results in guanine fragmentation, a critical step towards mutagenesis and carcinogenesis (Burney *et al.*, 1999; Niles *et al.*, 2006). Peroxynitrite can also nitrate guanine, yielding 8-nitro-guanine, which leads to formation of abasic sites and that can be cleaved *in vivo* by endonucleases to give DNA single-strand breaks (Burney *et al.*, 1999; Niles *et al.*, 2006). Peroxynitrite may also attack the sugar–phosphate backbone by abstracting a hydrogen atom from the deoxyribose moiety, resulting in opening of the sugar ring and generation of DNA strand breaks (Burney *et al.*, 1999; Niles *et al.*, 2006). The formation of DNA single-strand breaks represent a critical aspect of peroxynitrite-mediated cytotoxicity, since they represent the obligatory trigger for the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), a pathway ultimately related to the induction of cell death and tissue inflammation (Szabo *et al.*, 1996; Pacher and Szabo, 2008).

**Peroxynitrite and human diseases**

The hypothesis that peroxynitrite generation beyond body's defense contributes to myocardial and vascular dysfunction during ischemia and reperfusion, myocarditis, chronic heart failure and other cardiovascular pathologies has been the focus of intensive investigations during the last decade. Peroxynitrite may contribute to vascular pathophysiology by various mechanisms including triggering apoptosis and/or PARP-dependent cell death in endothelium

(Dickhout *et al.*, 2005; Mihm *et al.*, 2000) and vascular smooth muscle cells (Li *et al.*, 2003; Li *et al.*, 2004b) by inducing up-regulation of adhesion molecules in endothelial cells, endothelial glycocalyx disruption, enhancing neutrophils adhesion (Pacher et al., 2007), inhibiting voltage-gated $K^+$ K(v) and $Ca^{2+}$–activated $K^+$ channels in coronary arterioles (Li *et al.*, 2004a; Liu *et al.*, 2002) and vascular prostacyclin synthase (Pacher *et al.*, 2007). Furthermore, clinically relevant concentrations of 3-nitrotyrosine (present in various pathophysiological diseases) result in concentration-dependent impairment of acetylcholine-induced, endothelium-dependent vascular relaxation and may induce DNA damage in vascular endothelial cells ( Mihm *et al.*, 2000; Zou *et al.*, 2002).

There are four major mechanisms underlying the toxicity of peroxynitrite in shock. These include lipid peroxidation, depletion of antioxidant reserves (especially reduced GSH), oxidation/nitration of proteins (especially mitochondrial proteins) and finally, induction of DNA damage leading to the activation of poly (ADP-ribose) polymerase (Gonsette, 2008). In addition to direct cytotoxic effects of peroxynitrite such as peroxidation of lipids, proteins and DNA, peroxynitrite also occupies a critical position in a positive feedback loop of inflammatory injury, activating pro-inflammatory signaling and by triggering the recruitment of phagocytes within injured tissues, leading to further nitric oxide combine superoxide to form peroxynitrite, which will progressively amplify the initial inflammatory reactions. These observations point out that reduction of peroxynitrite or its precursors might have a considerable therapeutic impact in clinical circulatory shock.

**Autoimmunity**

It is a physiological inherent feature of the human immune system (Wardemann and Yurasov, 2003: Janeway *et al.*, 2005) defined by the presence of auto-reactive lymphocytes and antibodies. Development of the disease by transferring the active component of the immune response to an appropriate recipient (usually an animal model, or in some instances materno-fetal transfer of autoantibody) is the best proof that a disease is caused by autoimmunity. Autoimmune diseases can be organ specific (Hashimoto's thyroiditis, Thyrotoxicosis, Pernicious anaemia, Addison's disease, Insulin dependent diabetes

mellitus, etc) or non-organ specific (like Scleroderma, Sjogren's syndrome, Dermatomyositis, Systemic vasculitis, Systemic lupus erythematosus etc).

Evidence for genetic influence predisposing to autoimmunity is provided by studies on families. Moreover, many autoimmune diseases are linked to the major histocompatibility complex, which control a number of aspects of the immune response including antigen presentation. With one or two exceptions, however, the genes controlling predisposition to autoimmunity are heterogeneous within individuals and even within specific diseases. Of the environmental factors, infections appear to be the loss of tolerance to self-antigens (Wucherpfennig, 2001). Mechanisms whereby infections may cause autoimmunity include molecular mimicry; by the production of superantigens; via enhanced processing and presentation of autoantigens during an infection (epitope spreading); and by provoking bystander activation (Wucherpfennig, 2001). The potential ability of infectious agents to unlash the immune system to destructive attack by shifting it away from itself is known to include strategies of confusing the immune system by virtue of cross-reactivity or molecular mimicry (McClain and Heinlen, 2005) or by inducing polyclonal activation of B cells (Granholm and Cavalio, 1992) and possibly loss of functional suppressor T-cells (Janeway *et al.*, 2005).

**Mechanism of production of autoantibodies against protein antigens**

What triggers autoantibody production is a pivotal question to be answered in understanding the onset of autoantibodies-induced autoimmune diseases. Generally, majority of endogenous antigens are non-immunogenic due to immunological tolerance at the T cell and/or B-cell level. It has been suggested that self-proteins are rendered immunogenic if they are chemically modified post–translationally under various physiologic or pathologic conditions (Doyle and Mamula, 2001; Utz. and Anderson, 1998). In inflamed tissues or apoptotic cells, a variety of structurally modified proteins have been found (Doyle and Mamula, 2001; Piacentini and Colizzi, 1999; Ohmori and Kanayama, 2003). Such protein modifications may lead to the generation or unmasking of new antigenic epitopes that will serve to activate B-cells and/or T-cells, thereby impairing or bypassing immunological tolerance (Ohmori and Kanayama, 2005). The excluded B-cells are short-lived (1–2 days) and require stronger signals for activation than

non-auto-reactive B-cells, thereby decreasing the risk of autoimmune responses (Liu, 1997; Townsend *et al.,* 1999). Strict clonal deletion for establishing immunological tolerance may result in the formation of "holes" in the lymphocyte repertoire. Individuals with such "holes" will be susceptible to the attack by pathogens that possess self-mimicking epitopes. Thus, the leakiness of the central tolerance may be important to overcome the "hole problem" in the B-cell repertoire (Townsend *et al.,* 1999). If the auto reactive B-cells present in the periphery are activated, and recruited to the affinity maturation pathway, this may lead to generation of pathogenic autoantibodies (Kouskoff *et al.,* 2000; Mease, 2008).

Post–translational modification of self-proteins is a physiological mechanism of generating new antigenic epitopes and T-cell tolerance has been suggested to be broken or bypassed if new antigenic epitopes are created on self-proteins (Ohmori and Kanayama, 2005). Moreover, the reactivation of silenced self-reactive B-cells is believed to be one of the critical mechanisms triggering autoantibody production. Post–translational modifications include, for instance, transglutamination, isomerization to isoaspartyl, deamidation, deimination (citrullination), glycosylation, oxidation, phosphorylation, transglutaminase-mediated cross-linking, proteolytic cleavage by granzymes or caspases, oxidative fragmentation (Utz and Anderson, 1998; Amoura *et al.,* 1999; Casciola-Rosen *et al.,* 1997) and nitration. Some proteins listed in Table 1 have been found to generate neo-epitopes upon post-translational modifications.

Histochemical analysis of proteins from inflamed or infected tissues have revealed nitrotyrosine in their structure. Influenza virus-infected mice, alveolar macrophages and neutrophils were intensely stained with anti-nitrotyrosine antibodies (Akaike *et al.,* 1996). Macrophages obtained from surgical resection of lung & colonic cancers also contain nitrotyrosine-immunopositive proteins in the cytoplasm (Brito *et al.,* 1999). IgG antibody against nitrotyrosine may be formed *in vivo* under inflammatory conditions because nitrotyrosine–IgG complex have been reported in synovial fluids of rheumatoid arthritis patients (Uesugi *et al.,* 2000). It has been suggested that nitrotyrosine may serve as a B-cell epitope because both polyclonal and monoclonal anti–nitrotyrosine antibodies have been prepared by

immunization with nitrotyrosine-bearing heterologous proteins (Ye *et al.*, 1996). An interesting feature of nitrotyrosine is its structural similarity to a synthetic hapten called 4-hydroxy-3-nitrophenylacetyl (NP). Anti-dsDNA antibodies from lupus mice are generally polyreactive (Radic and Weigert, 1994; Eilat and Naparstek, 1999) and have been found to cross-react with NP-related haptens (Zouali *et al.*, 1987).

**Table 1**

**List of proteins which have given birth to new antigenic epitopes after post–translational modification reactions**

| Modification reaction | Substrate protein | Reference |
|---|---|---|
| Transglutamination | Histone H2B, actin, myosin, keratins | Doyle and Mamula, 2001 |
| Isoaspartylation | snRNP | Doyle and Mamula, 2001 |
| Deamidation | Wheat gliadin | Sollid, 2000 |
| Deimination (citrullination) | Myelin basic protein, fibrin | Cao *et al.*, 1998 |
| Glycosylation | Type II collagen | Corthay *et al.*, 1998 |
| Oxidation | LDL | Shoenfeld *et al.*, 2001 |
| Phosphorylation | Pre-mRNA splicing factors αβ-crystallin | Doyle and Mamula, 2001 |
| Tyrosine nitration | Multiple proteins in inflamed tissues | Ischiropoulos, 1998 |

## Autoimmune diseases

Autoimmune diseases are those which result from immunologic reactions, humoral and/or cellular or a combination of both. These diseases may be classified in different ways; it may be organ/tissue specific such as Hashimoto's thyroiditis, Thyrotoxicosis, Pernicious anaemia, Addison's disease, Insulin dependent diabetes mellitus, Myasthenia gravis etc. (Bodil *et al.*, 2008; Bizzaro, 2008) or non–organ specific (systemic) such as autoimmune haemolytic anaemia, Systemic lupus erythematosus, Idiopathic thrombocytopenia purpura, Autoimmune leucopenia, Chronic active hepatitis, Coeliac disease, Autoimmune enteropathy, Autoimmune polyendocrinopathy, Sjogren's syndrome, Dermatomyositis, Systemic vasculitis,

Anti-phospholipid syndrome etc. (Pittock *et al.*, 2008). No single theory or mechanism can adequately explain all the features of autoimmune diseases. However, three models have been proposed for the induction of a productive autoimmune process. The first is the autoimmune response to a cross-reacting antigen, i.e. cross reactivity between host and foreign antigens or modification of host proteins due to infection, drug administration and inflammation etc. (Theofilipoulos and Dixon, 1985; Huang *et al.*, 1988). The second is the genetic predisposition for autoimmunity through regulatory failure and the third is the triggering with bacterial mitogen of potentially autoimmune B-cell which normally held in check by suppressor cells.

Autoantibodies are commonly regarded as being intrinsically involved in the tissue injury associated with autoimmune diseases. Whilst this may be true in a number of important examples (e.g. Systemic lupus erythematosus, Goodpasture's disease and Myasthenia gravis), this is not true of all autoimmune diseases. For example, autoantibodies may form secondary to the tissue damage associated with the underlying disease (e.g. cardiac antibodies produced after myocardial infarction) (Katsorida and Moutsopoulos, 2001). Autoantibodies may also occur as an epiphenomenon to the underlying disease, and whilst in this situation they may be a useful marker for the presence or predisposition to certain autoimmune processes, they are not in themselves directly contributing to tissue injury. Table 2 summarizes the most commonly encountered autoantibodies with their disease associations.

## Genetic factors in autoimmunity

Clinicians treating patients of autoimmune disorders have long been struck by the findings that these patients frequently have relatives suffering from the same or different autoimmune disorders (Shoenfeld and Isenberg, 1989). Several chromosomal regions/genes have been defined that could confer generic liability to autoimmune diseases (Tsao, 2003). Majority of the genes associated with autoimmune diseases, within the major histocompatibility (MHC) class II alleles, display various abilities to bind and present peptides to lymphocytes (Marrack and Kappler, 2001). Some alleles might be more predisposing to autoimmunity. Specific alleles of MHC class II genes have been linked with SLE in ethnic groups,

e.g. increased frequency alleles encoding for HLA-DR3 was observed in Caucasians and in Japanese lupus patients stronger association with HLA-DR2 were reported (Hahn, 2005).

**Table 2**

**Autoimmune diseases and their respective autoantibodies**

| Disease | Autoantibodies directed against |
|---|---|
| **Non-organ specific** | |
| Systemic lupus erythemetosus | Nuclear antigens, DNA, Sm, Cardiolipin |
| Scleroderma | Nuclear antigens, Scl-1, Scl-70 |
| Sjogren's syndrome | Nuclear antigens SS-A, SS-B |
| Mixed connective tissue disease | Extractable nuclear antigen (RNP) |
| Rheumatoid arthritis | Fc region of IgG (rheumatoid factor), Nuclear antigens |
| Chronic active hepatitis | Smooth muscle antigen |
| Autoimmune haemolytic anaemia | Red cell membrane antigens |
| Multipule sclerosis and related demyelinating diseases | Myelin basic protein antigen |
| **Organ specific** | |
| Autoimmune thyroid diseases | Thyroglobulin, Microsomal antigen, TS1 |
| Addison's disease | Aderanal cortical cell antigen |
| Pernicious anemia | Intrinsic factor, Parietal cell antigen |
| Inflammatory bowel diseases | Colonic mucosal antigen |
| Primary biliary cirrhosis | Mitochondrial antigen |
| Myasthenia gravis | Acetylcholine receptor |
| Goodpasture's syndrome | Glomerular basement membrane |
| Pemphigus vulgaris | Epidermal intercellular substance |
| Bullous pemphigold | Skin basement membrane |
| Insulin dependent diabetes | Islet cell antigen (cytotoxic T-cells) |
| Wegener's granulomatosis and other vasculitides | Serine protease and myeloperoxidase |

The HLA system plays a major role in the normal functioning of the immune system. Hundreds of associations between HLA alleles and autoimmune disorders (as well as other diseases) have been found (Shiina *et al.*, 2004).

**Immunological factors in autoimmunity**

The immuno-regulatory network plays major role in the onset and development of autoimmune diseases. Abnormality in the immune system causes reaction against surface antigens. Role of B- and T-cells and inhibitory molecules have been suggested in the initiation of autoimmune diseases although self-reactive B- and T-lymphocytes are eliminated during their development in the bone marrow or thymus by negative selection, and the process is called central tolerance (Lequn and Vassiliki, 2006). B-cells also function as antigen presenting cells (APCs) and may present its own antigens, through MHC, to T-cells as foreign bodies. As a result, the B-cell receives signals from the activated T-cell which induces autoantibodies. The abnormal immune response probably depends upon interactions among susceptibility genes and numerous environmental factors. In wide variety of autoimmune diseases, the regulatory failure results in significant decrease in T-suppressor cell members and their activity, thereby unbalancing the T-helper/T-suppressor ratio. The increased helper/suppressor ratio has been observed in SLE, Sjogren's syndrome, Scleroderma, Rheumatoid arthritis, Pernicious anemia, Multiple sclerosis, Immune complex mediated renal diseases, Immunologic skin disease etc. Furthermore, immunologic cross reactivity and molecular mimicry is an important phenomenon in autoimmune diseases (Prinz, 2004).

**Environmental factors in autoimmunity**

Infections agents, medications, chemicals, toxins and ultraviolet light have been implicated in autoimmune disorders (Saraux *et al.*, 1999). Ultraviolet light is a known trigger for SLE (McGrath, 1999). D-penicillamine and hydralazine, have been implicated in the development of SLE and glomeruloephritis (Brik *et al.*, 1995). Exposure to mercury could induce lupus like features (Parks and Coper, 2005).

## Rheumatoid arthritis

It is a chronic systemic inflammatory condition of unknown cause that affects approximately 0.8% of the world population. Rheumatoid arthritis (RA) is a multifactorial disease frequently characterized by synovial inflammation usually involving peripheral joints, cartilage destruction, bone erosions and subsequent joint deformity. The synovium of the inflamed joints is invaded by T-lymphocytes expressing an activated genotype and phenotype, as well as neutrophils, monocytes, B lymphocytes and dendritic cells (Ruddy *et al.,* 2005). Women are more susceptible (three times) to RA than men and the peak incidence is between 40 and 60 years, but men and women of all ages may be affected. The exact cause of RA remains obscure, but it is thought to be triggered and initiated by bacterial or/and viral infection(s). Numerous studies on RA patients have revealed increased synthesis of endogenous nitric oxide. A number of cells within the joint; including endothelial cells of synovial capillaries, mesenchymal cells, neutrophils, fibroblasts, lymphocytes, mast cells and macrophages; are able to generate substantial quantities of nitric oxide (Van't Hof and Ralston, 2001) which rapidly reacts with superoxide radical to give peroxynitrite (Huie and Padmaja, 1993). The formed peroxynitrite can be directly cytotoxic else, it can decompose to give hydroxyl radical and nitronium ion. Here, it may be pointed out that addition of peroxynitrite to biological fluids lead to nitration of aromatic amino acid residues, and their presence may be 'marker' of peroxynitrite-mediated ($NO^{\bullet}$– dependent) damage *in vivo* (Kaur and Halliwell, 1994). Overproduction of peroxynitrite may be important in the pathogenesis of RA.

Some investigators have reported a significant correlation between serum nitrate and number of tender joints, number of swollen joints and ritchie articular index (Onur *et al.,* 2001). Peroxynitrite produced in the inflamed joints may contribute to the peri-articular bone-loss observed in RA (Van't Hof and Ralston, 2001). In many joint diseases, pro-inflammatory factors such as cytokines and prostaglandins are released at sites of inflammation along with ROS (Henrotin *et al.,* 2003) and nitric oxide (Sakurai *et al.,* 1995). These factors are associated with very low concentration of superoxide dismutase in joint fluid (Marklund *et al.,* 1986). Studies involving assay of nitrotyrosine residues in synovial tissues of

RA patients (Sandhu *et al.*, 2003), or exposure of chondrocytes to synthetic peroxynitrite *in vitro* (Mathy-Hartert *et al.*, 2003) have established that combination of superoxide to nitric oxide causes cartilage damage. Studies on animal models have revealed that inhibition of peroxynitrite synthesis may have a therapeutic role in RA (McCartney- Francis *et al.*, 1993). A traditional herbal remedy tripterygium wilfordii Hook F (TWHF) have also been reported to be effective in the treatment of rheumatoid arthritis (Wang *et al.*, 2004). The major active component of TWHF, triptolide, significantly inhibited nitric oxide production in activated peritoneal macrophages and decreased the level of iNOS mRNA, suggesting that the specific inhibition of the iNOS gene may be responsible for the anti-inflammatory effects of TWHF. Transcription of the iNOS gene is activated by pro-inflammatory cytokines such as IL-1 and TNF-alpha whereas glucocorticoids are inhibitory, thereafter peroxynitrite generation can be inhibited with anti-TNF (by reducing iNOS) as well as directly, by inhibiting iNOS activity.

Furthermore, several studies have confirmed the reduced level of androgens and progesterone in RA patients. Men suffering from RA have been reported to possess low testosterone, dehydroepiandrosterone and estrone while estradiol, a naturally occurring estrogen, was found to be increased which correlated well with the inflammatory indices (Tengstrand *et al.*, 2003). Synovial fluids of both male and female patients of RA contain significantly high level of estrogen (in comparison to androgens) due to increased aromatase activity mediated by TNF-α (Cutolo *et al.*, 2006). Not only the increased estrogen level, but also the differential expression of estrogen receptors on immune cells might be implicated in the pathologic changes seen in the joints of RA patients. Although sex hormones might be implicated in the pathogenesis of RA, their role seems much less critical for the disease development and flares than in systemic lupus erythematosus. This notion is supported by the fact that RA develops predominantly at age 40 and above, and that female patients experience remissions during pregnancy when estrogenic hormones reach their peak. In collagen induced arthritis (CIA), the disease was shown to be accelerated and much more severe in male mice than their female

counterparts (Wilder, 1996). Subsequent treatment with estradiol decreased inflammatory cytokines (TNF-α and IL-1) and ameliorated the disease activity.

RA may be accompanied by many autoantibodies in patients' sera. Most of these antibodies are not specific for RA because they also occur in other inflammatory conditions. Other antibodies appear to be more specific and are, in some cases, almost exclusively present in RA. The possible pathogenic nature of autoantibodies in RA is still controversial (Smolen and Steiner, 1998). The presence of autoantibodies that react with the Fc portion of IgG (rheumatoid factor) is currently used as a marker for RA (Tighe and Carson, 1997). However, rheumatoid factors have modest specificity (~70%) for the disease. In recent years, several newly characterized autoantibodies have become promising candidates as diagnostic indicators for RA. Antikeratin (Cordonnier *et al.*, 1996), anticitrullinated peptides (Schellekens *et al.*, 2000), anti-RA33 (Hassfeld *et al.*, 1995), anti-Sa (Hueber *et al.*, 1999) and anti-p68 (Blass *et al.*, 2001) autoantibodies have been shown to possess >90% specificity. Antinuclear antibodies are found in approximately 50% of RA patients (Steiner and Smolen, 2002). With the exception of anti-RA33 and antibodies to Epstein-Barr virus nuclear antigen, specific antinuclear antibody subsets among patients with connective tissue diseases are very rare. Anti-Ro may occasionally be present in RA patients, especially if they suffer from secondary Sjögren's syndrome.

**Systemic lupus erythematosus**

It is a multisystem autoimmune disease with protean clinical manifestations that may affect any organ or system in human or experimental animals. The disease is characterized by flares, remissions and autoantibodies to several intracellular and cell-surface antigens. SLE is found worldwide with an estimated prevalence of 12–64 per 100,000 populations. The incidence is higher in urban populations. In most patients, SLE develops between 15 and 45 years of ages. The female:male ratio is at least 9:1. In a minority of patients, disease develops after the age of 50 years, and in this subgroup the female:male ratio is 4:1.

Numerous investigators have attempted to identify candidate genes in SLE, but many implicated genes have not been confirmed. Current evidence suggests a role for the HLA region (i) complement components (ii) and IgG receptors (iii).

(i) The HLA alleles; A1, B8, DR2 and DR3 have been shown to be associated with SLE in Caucasian populations.

(ii) Deficiencies in classical pathway complement components C1q, C2 and C4 are strongly associated with the development of a lupus-like disease.

(iii) IgG receptors on mononuclear phagocyte cells are responsible for clearing IgG and IgG-containing immune complex from the circulation. In Afro-American patients, associations have been found between SLE and the presence of low-affinity IgG receptors.

Multiple autoantibodies, typically antinuclear and anti-DNA antibodies characterize SLE (Tan, 1989; Davidson *et al.*, 1990; Steinberg, 1992; Harada *et al.*, 1994; Petri, 1996). The clinical manifestations include fever, an erythematosus 'butterfly rash' across the face, lesions of discoid lupus or vasculitis rash, malar rash, discoid rash, photosensitivity, oral ulcers, non-erosive arthritis, pleuritis or pericarditis, anemia, thrombocytopenia, renal, neurologic and cardiac abnormalities. One of the impediments in understanding human systemic lupus erythematosus has been its marked heterogeneity (Steinberg *et al.*, 1991; Steinberg, 1992). Evidence suggests that the initial autoimmune response in SLE is restricted to few epitopes (Sing, 2004). The phenomenon of epitope spreading offers an explanation for the development of autoantibody specificities to antigens that are part of the same molecular complex, such as the close association of anti-Ro and anti-La antibodies (Weistein *et al.*, 2004).

The initial immunizing antigens that drive the development of SLE are unknown, but characteristics of the immune response in SLE suggest that it is an antigen-driven condition. These include oligoclonal expression of antigen-specific cells, up-regulation of parallel B- and T-cell responses against the same antigen, epitope spreading to progressively more diversified structures on the antigen and selection for immune cells with higher affinity for the antigen (Greidinger, 2001).

Apoptosis play a crucial role in regulation of immune system. Impaired apoptosis may be caused by deficiencies in endogenous pro-apoptotic mediators, over-expression of endogenous pro-apoptotic mediators or by acquired factors with increased risk for the development of SLE. In SLE, the immune system attacks various auto-antigens and cause damage in target organs. It has been suggested that

dead cells may serve as a repertoire for auto-antigens which can stimulate an autoimmune response.

Role of sex hormones in autoimmunity has been most extensively evaluated in SLE patients. The preponderance of SLE in young woman of child bearing age with a female to male ratio of 9:1 and the tendency for lupus flares during pregnancy and remissions after menopause or cyclophosphamide induced ovarian failure; suggest that female sex hormones are crucial regulators of lupus activity (Peeva and Zouali, 2005). Hormones influence the sexual dimorphism of the immune system. They can initiate or accelerate an autoimmune process and thereby contribute to gender biased autoimmune disorders. The female sex hormones, estrogen and prolactin, are considered immunomodulators and have been implicated in autoimmunity. Not only endogenous estrogens, but also environmental estrogens may act in conjunction with other factors to override immune tolerance to self-antigens (Peeva and Zouali, 2005). In the B-cell compartment, both prolactin and estrogen are immuno-stimulators that affect maturation and selection of auto-reactive B-cells, as well as autoantibody secretion, while progesterone is an immuno-suppressor. The impact of prolactin and estrogen may be based on their capacity to allow auto-reactive B-cells to escape the normal mechanisms of tolerance and mature to fully functional antibody-secreting B-cells that can cause clinically apparent lupus. However, the mechanism of action of the two hormones is different; estrogen leads to the survival and activation of auto-reactive B-cells with a marginal zone phenotype, whereas prolactin induces self-reactive B-cells with a follicular zone phenotype (Grimaldi *et al.*, 2005).

Deficiencies in the classical pathway of the complement system have been implicated in the etiology and pathogenesis of SLE (Liu *et al.*, 2004). Complement has both beneficial and deleterious roles in the pathogenesis of SLE. Decreased levels of C1, C2, C3a and C4 complement in SLE and their deposition in inflamed tissues is suggestive of harmful role of complement in the effectors phase of disease. However, homozygous deficiencies of any of the classical pathway proteins are strongly associated with the development of SLE. There are two main hypotheses to explain these observations. The first invokes unimportant role for

complement in the physiological waste-disposal mechanisms of dying cells and immune complexes. The second hypothesis is based around the role of complement in detraining the activation threshold of B- and T-lymphocytes with the proposal that complement deficiency causes incomplete maintenance of peripheral tolerance (Manderson *et al.*, 2004). Excessive complement activation as a result of a regulator component deficiency leads to tissue injury. Natural antibodies and probably autoantibodies present in the sera of patients with systemic autoimmune diseases bind to tissues already exposed to damaging insult, activate complement and produce pathology (Tsokos and Fleming, 2004). Polymorphisms in low affinity IgG (Fcy) receptors, which are important for the clearance of immune complexes, are also implicated in the pathogenesis of lupus (Salmom *et al.*, 1996; Wu *et al.*, 1997).

The source of the antigens that drive B-cell responses in SLE is unknown, although studies suggest mechanisms by which the self-antigens become immunogenic and stimulate responses. In addition to reflecting increased exposure to self-antigens, autoantibody responses in SLE may result from abnormalities in B-cell signaling and regulation by cytokines (Criscione and Pisetsky, 2003). B-cell activation generally requires T-helper cells. T-cells cloned from lupus prone mice were found to stimulate the production of anti-DNA antibodies and renal lesion when injected. Obligatory and enhanced T-cell help for B-cell has been shown in SLE lymphocytes by prolonged expression and co-stimulatory interaction of the helper T-cell surface ligand CD40 (CD40L) with B-cell receptor (CD40B) (Desai *et al.*, 1996; Koshy *et al.*, 1996). Interaction of CD40L on activated T-cells with CD40B cells induces B-cell proliferation and formation of germinal centers. Further cell to cell interactions within germinal center lead to B-cell interaction through immunoglobulin isotype switching, somatic mutation, clonal expression of high affinity B-cells and terminal differentiation to plasma cells (Reiser and Stadecker, 1996; Lindhout *et al.*, 1997; Tarlinton *et al.*, 1998). Blockade of CD40L has been shown to delay the onset of disease in SLE-prone mice and to stabilize or reverse existing renal disease (Mohan *et al.*, 1995; Kalled *et al.*, 1998). Further studies in SLE-prone mice showed that an anti-CD40L monoclonal

antibody administered to prenephritic mice inhibit both T-cell activation and T-cell dependent B cell activation (Huang *et al.*, 2002).

**Peroxynitrite – inflammation nexus**

Inflammation stands in the foreground of a large number of chronic conditions. These include rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis and systemic lupus erythematosus. Potential biological targets of peroxynitrite include membrane as well as cytosolic and nuclear receptors. Putative targets of peroxynitrite include epidermal growth factor receptor, the $\beta_1$- and $\beta_2$-adrenoceptors (ADRB1 and ADRB2), platelet/endothelial cell adhesion molecule1 (PECAM1), insulin receptor substrate 1 (IRS1) and peroxisome proliferator-activating receptor gamma (PPARγ) (Pacher *et al.*, 2007). The peroxynitrite-mediated oxidation and/or nitration of these receptors may produce disturbances in downstream signalling. The reaction of peroxynitrite with fibroblast growth factor-1 induces extensive cysteine oxidation, tyrosine nitration and irreversible inactivation of protein activity (Bagnasco *et al.*, 2003). Peroxynitrite may profoundly influence inflammatory responses at multiple levels. During inflammation, which provides a setting that favors the biological formation of peroxynitrite, reactions of peroxynitrite with inflammatory mediators such as interleukins (ILs) and inducible NOS (iNOS) may provide means for regulating the inflammatory response. In contrast to IL-8, peroxynitrite was found to enhance the anti-inflammatory function of IL-10 (Freels *et al.*, 2002) and decrease the activity of iNOS (Lanone *et al.*, 2002), which could be viewed as a negative-feedback mechanism for $NO^{\bullet}$ and peroxynitrite formation during inflammation. In human neutrophils, peroxynitrite triggers the down regulation of L-selectin expression and the upregulation of CD11b/CD18 expression (Zouki *et al.*, 2001). These effects are likely to be mediated, at least in part, by the ability of peroxynitrite to trigger and enhance nuclear factor-κB (NFκB)-mediated pro-inflammatory signal-transduction pathways (Matata and Galinanes, 2001) by modifying proteins that are associated with the activation of this transcription factor (Bar-shai and Reznick, 2006; Zouki *et al.*, 2001; Matata and Galinanes, 2001; Levrand *et al.*, 2005). Additional pro-inflammatory actions of peroxynitrite may involve its capacity to serve as a cyclooxygenase (COX) substrate (Landino *et al.*, 1996), although larger

concentrations of the oxidant may inactivate the enzyme (Trostchansky *et al.*, 2003).

Furthermore, peroxynitrite can also contribute to the enhanced production of pro-inflammatory mediators by decreasing histone deacetylase-2 (HDAC2) activity through nitration (Ito *et al.*, 2004). Overall, elevated local levels of peroxynitrite during inflammatory conditions may lead to a deregulation of cellular signal-transduction pathways (Levonen *et al.*, 2001), upregulation of localized inflammatory stress response and possibly promotion of cellular and tissue injury. The upregulation of adhesion receptors by peroxynitrite may also result in an increased expression of endothelial adhesion molecules and such cells might represent a preferential site for the adhesion and migration of neutrophils when high concentrations of nitric oxide and neutrophil-derived superoxide are simultaneously present (Sohn *et al.*, 2003).

**Anti-histone autoantibodies in SLE and RA**

Histones are small, highly conserved, extremely cationic and DNA binding proteins (Rubin, 1989). They are the major constituent of chromatin and may be found in circulation as nucleosomes (Schmiedeke *et al.*, 1989). Autoimmune diseases such as systemic lupus erythematosus (SLE), reumatoid arthritis (RA) are characterized by autoantibodies reactive to a wide range of nuclear and cytoplasmic antigens including DNA, RNA, histones, non-histone proteins, surface molecules and intracellular matrix proteins (Theofilopoulos and Dixon, 1981; Smolen and Steinberg, 1982). Antinuclear antibodies (ANAs) can penetrate into viable cells and interact with nuclear antigens. Indeed, intranuclear immunoglobulin may occasionally be found in live circulating Fcγ+ mononuclear cells from SLE patients or other autoimmune diseases (Fishbein *et al.*, 1979). Because the interaction of ANAs with different nuclear antigens *in vivo* could result in different pattern of circulating dysfunction, it seems important to determine the clinical characteristics of SLE patients with different antinuclear antibodies. Some investigators attribute the diversity of autoantibodies in these diseases to generalized (polyclonal) activation of immunoglobulin G-producing B-cells (Klinman and Steinberg, 1987). Other believe that autoreactive clones are specially and preferentially stimulated remain quiescent (Hardin, 1986).

Autoantibodies to histones are found in patients with SLE and RA but their role in pathogenesis is unclear (Harley *et al.*, 2004). The specificity of autoantibodies against H1, H2A, H2B, H3 and H4 histones in SLE have been found to be 60, 53, 48, 38 and 30 percent respectively (Ghedira *et al.*, 2006; Sun *et al.*, 2008). Antihistone antibodies (AHAs) are among the most prevalent antinuclear antibodies most frequently found in drug induced lupus or SLE. Because of histones' affinity for glomular basement membrane (Schmiedeke *et al.*, 1989) and cell surface (Jacob *et al.*, 1989) it has been suggested that histone-antibody complex might play an important pathogenic role in SLE (Bustos *et al.*, 1994). Among anti-histone autoantibodies, antibodies to the H2A-H2B complex and (H2A-H2B)-DNA complex have been found to be more sensitive and specific marker for SLE than autoantidodies to individual histones (Hasegawa *et al.*, 1998). Antibodies against histones are heterogenous, some react with the isolated histones, other react with DNA-histone complex (Winfield *et al.*, 1978). Optimal antibody response to most native proteins requires the interaction of antigen with accessory cells, helper T-cells and B-cells (Singer and Hodes, 1983; Julius *et al.*, 1982). Although helper T-cells recognize denatured forms of antigen in association with class II major histocompatibility complex molecules. B-cells react via surface immunoglobulin (Ig) receptors with mostly unprocessed determinants (Partanova *et al.*, 1987). It has been indicated that the determinant recognized by B-cells are not distributed randomly with particular structural properties (Berzofsky, 1985). These are hydrophilic regions present on the outer surface of the molecules and are generally not restricted in mobility by secondary intra– or inter–molecular interactions.

For anti-histone antibodies spontaneously produced in SLE, the major autoantigenic determinants were mapped to be the amino and carboxyl terminal regions of histone (Gohill *et al.*, 1985). Such regions are expected in nucleosome core and are preferentially digested when chromatin is treated with trypsin (Sollner-webb *et al.*, 1976). On the basis of complement fixation assay, anti–histone antibodies in patients with systemic rheumatoid diseases are thought to occur relatively infrequently and in low titer (Stollar, 1971). Several investigators have pointed out that anti-histone antibodies detected by using this assay are

present in the least 30% of the patients with idiopathic SLE and 35% of patients with rheumatoid arthritis (Aitcheson *et al.,* 1980). Later, it was determined that idiopathic SLE sera reacted with all histone (H1, H2A, H2B, H3 and H4), although the majority reacted with the bimolecular complex of H2A and H2B (Gohill *et al.,* 1985).

**Nitrotyrosine in systemic lupus erythematosus and rheumatoid arthritis**

In lupus, a correlation between serum nitrate/nitrite and nitrotyrosine has been observed. Although both are measure of nitric oxide production but not necessarily correlated with individual patients. Serum nitrate/nitrite is a metabolite of nitric oxide and peroxynitrite and is rapidly cleared from the circulation. Thus, they reflect systemic peroxynitrite production at that time, similar in a way to measures of serum glucose in diabetes. Nitration of tyrosines, however, is irreversible and can be detected in *in vivo* as nitrated proteins. Serum nitrotyrosine levels reflect nitric oxide production over a period of time, perhaps most analogous to serum glycated hemoglobin in diabetes (Gilkeson *et al,* 1998).

Nitrated renal proteins have been found in murine lupus nephritis, which correspond with increase nitrated serum proteins and abnormal staining of nitric oxide synthase-2 (NOS2) enzyme in the kidney (Wang *et al.,* 1991). Abnormal NOS-2 expressions in keratinocytes and vascular endothelium of lupus patients have been observed. Wang *et al.,* 1991 reported increased apoptotic activity among renal cells adjacent to those expressing NOS-2 protein. Any or all of above mechanisms may contribute to NO-mediated damage in lupus. Free 3-nitrotyrosine and serum nitrate/nitrite are rapidly cleared, but protein 3-nitrotyrosine should be retained in those subjects without protein urea. However, elevated serum 3-nitrotyrosine has been seen in subjects with proteinurea despite increased protein loss. Thus, serum 3-nitrotyrosine may serve as a measure of protein modification by nitric oxide/peroxynitrite (Oates *et al.,* 1999; Hardy *et al.*, 2008).

**Objectives of the present study**

Our major aim was to study the impact of laboratory synthesized peroxynitrite on the structure of histones and to test whether the peroxynitrite modification had any bearing on the immunogenicity of H2A histone. Structural changes were monitored by UV-visible spectroscopy, fluorescence, CD, FT–IR

spectroscopy, HPLC and SDS-polyacrylamide gel electrophoresis. Immunogenicity was evaluated by injecting native and peroxynitrite-modified H2A histones into experimental animals.

Our second aim was to evaluate the binding profile and specificity of naturally occurring autoantibodies in systemic lupus erythematosus and rheumatoid arthritis patients with native and peroxynitrite-modified H2A histones, with an intention to establish or define the role of peroxynitrite-modified H2A histone in systemic lupus erythematosus and rheumatoid arthritis. The antigen-antibody interaction was studied by direct binding & inhibition ELISA and mobility shift assay.

# Material & Methods

## MATERIAL

Calf thymus histones (H1, H2A, H2B and H3), 3-nitrotyrosine, uric acid, ascorbic acid, vitamine E, dithiothreitol (DTT), catalase, superoxide dismutase (SOD), glutathione (reduced), cysteine, calf thymus DNA, bovine serum albumin (BSA), nitrated BSA, anti-human/anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, tween-20, coomassie brilliant blue (G-250 & R-250), sodium dodecyl sulphate. Freund's complete and incomplete adjuvants, agarose, bisacrylamide, 1-anilinonaphthalene-8-sulfonic acid (ANS) and Protein-A agarose were purchased from Sigma Chemical Company, USA. Milex syringe filters (0.45 μM) were purchased from Millipore Inc., USA. Folin-Ciocalteau reagent was purchased from Centre for Biochemical Technology, New Delhi. Silver nitrate, formaldehyde, methanol, glacial acetic acid, iso-propanol, sodium chloride, sodium carbonate, sodium bicarbonate, magnesium chloride, potassium chloride, guanidinium chloride, sodium hydroxide, EDTA (disodium salt), hydrogen peroxide, sucrose and ammonium persulphate were obtained from Qualigens, India. Flat bottom polysorp ELISA modules were purchased from NUNC, Denmark. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N′,N′-tertramethylethylenediamine (TEMED) were from Bio-Rad Laboratories USA. All other reagents/chemicals were of the highest analytical grade available.

### Collection and processing of blood samples

Verbal consent of patients as well as healthy subjects was obtained before taking blood samples. Also, they were clearly told that the blood samples would be used only for the purpose of research. All SLE samples (n=50) used in this study were obtained from female patients (in the age group of 35-75 years) attending the medicine outdoor clinic and /or those admitted in the medicine ward of the All India Institute of Medical Sciences, New Delhi. The patients satisfied the criteria of SLE diagnosis established by the American College of Rheumatology (Arnett *et al.*, 1988) and showed high titre anti-dsDNA autoantibodies by enzyme immunoassay. Rheumatoid factor positive sera of arthritis patients (n=50) were collected from the Department of Microbiology located in the premises of the medical college of Aligarh Muslim University, India. All serum samples were decomplemented by heating at 56°C for 30 min.

## Equipments

Spectrophotometer (Shimadzu, Japan) with programmable melting device, spectrofluorometer (Shimadzu, Japan), microplate reader, Elico pH meter, polyacrylamide gel electrophoresis assembly (Genei, India), table top high speed refrigerated ultracentrifuge (Beckman, USA), HPLC–Duo flow (Bio-Rad Laboratories, USA), C18 HPLC column (Supelco discovery), spectropolarimeter (Jasco, USA) attached to a microcomputer and FT-IR Spectrometer (Bruker Vector 22, Germany) were the major equipments used in this study.

# METHODS

## Polyacrylamide gel electrophoresis (PAGE)

Banding patterns of proteins were analyzed by electrophoresis in polyacrylamide gel according to the procedure descried by Laemmli (1970). Slight modifications were made in the original protocol only to improve the separation and visualization of bands.

(i) **Acrylamide–bisacrylamide (30:0.8):—** A stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm bis-acrylamide in distilled water to a final volume of 100 ml.

(ii) **Resolving gel buffer:—** A stock solution was prepared by dissolving 36.3 gm Tris base in 48.0 ml of 1N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(iii) **Electrode buffer:—** 3.03 gm Tris and 14.4 gm glycine were dissolved in distilled water and pH was adjusted to 8.3. SDS (1.0 g) was added and volume made up to one litre.

(iv) **Procedure for making polyacriamide gel:—** Thoroughly cleaned glass plates, separated by 1.5 mm thick spacer, were sealed from bottom and sides with 1% agarose. The resolving gel mixture (10 % ) was prepared by mixing the components and poured into the space between the glass plates and allowed to solidify. Protein samples mixed with sample dye (10 % glycerol, 2 % SDS, 0.5 M Tris pH 6.8 and 0.002 % bromophenol blue) were placed into wells and electrophoresis was carried out for 3 to 8 hr at 50–80V (depending on the need). The electrophoresed proteins were visualized by staining with silver nitrate.

**Recipe for 10% resolving gel:—** Following solutions were mixed and immediately poured between the glass plates.

| Component | Volume |
|---|---|
| Acrylamide – bisacrylamide | 3.3 ml |
| Resolving gel buffer | 2.5 ml |
| Distilled water | 4.0 ml |
| 10% SDS | 0.1 ml |
| 1.5% ammonium persulphate | 0.1 ml |
| TEMED | 4.0 µl |

## SDS-PAGE under non-reducing conditions

Native and peroxynitrite-modified histones' samples were subjected to electrophoresis on 10 % polyacrylamide gel. The samples were mixed with one-tenth volume of sample dye and electrophoresed at 80 V for 3-4 hr in Tris-glycine (pH 8.3) buffer containing SDS. Protein bands were visualized by staining with silver nitrate.

## Silver nitrate staining of polyacrylamide gel

The procedure described by Merril *et al.*, (1981) was followed. Soon after electrophoresis, the protein bands were fixed by rapidly immersing the gel in a mixture, containing 40% methanol and 13.5% formaldehyde, for 15 min with occasional shaking. The gel was then washed with distilled water twice and transferred to 0.02% sodium thiosulphate ($Na_2S_2O_3$) solution and incubated for 2 min. Again, the gel was rinsed twice with distilled water. This was followed by treatment with 0.1% $AgNO_3$ solution for 20 min. The gel was washed with distilled water briefly and then immersed in a developer solution (3% sodium carbonate solution containing 0.5% formaldehyde and 0.02% $Na_2S_2O_3$) for 15 min or until the gel was properly stained. The reaction was stopped by transferring the gel to a stopper solution (25% isopropanol solution containing 10% glacial acetic acid) and left for 5 min. The gel was finally stored in distilled water.

**Preparation of histone solutions**

Commercially available histones (H1, H2A, H2B and H3) were dissolved in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl) and kept in aliquots at 4°C. As far as possible the histone solutions were protected from direct exposure to light.

**Laboratory synthesis of peroxynitrite**

Peroxynitrite was synthesized by rapid quenched flow process (Beckman *et al.,* 1996). Briefly, solutions of sodium nitrite (0.6 M in 50 mM sodium phosphate buffer, pH 7.4), hydrogen peroxide (0.6 N in 0.7 N HCl) and sodium hydroxide (1.2 M in distilled water) were prepared and chilled at 0°C for few minutes prior to use. The schematic representation of laboratory ware that were assembled to synthesize peroxynitrite is shown in Fig.1. A flow rate of 10 ml/min was adjusted to get most concentrated solution of peroxynitrite. The peroxynitrite was collected in 1.2 M NaOH and kept at -20°C. The concentration of stored peroxynitrite was assessed every time it was used; by recording absorbance at 302 nm and using molar extinction coefficient of 1670 $M^{-1}cm^{-1}$ (Hughes and Nicklin , 1968).

**Modification of histones by peroxynitrite**

The modification was carried out by incubating 25 μM of histones (H1, H2A, H2B and H3) with 100 μM of diethylene triamine penta–acetic acid (metal ion chelator) and 50 μM, 100 μM and 200 μM peroxynitrite at 37°C for 30 min. The approximate pH of the incubation mixture was in the range of 10-11.

**Spectroscopic analysis of native and peroxynitrite–modified histones**

*(a)* The absorption spectra of native and peroxynitrite–modified histones were recorded on spectrophotometer in the wavelength range of 250-500 nm using quartz cuvette of 1 cm path length.

*(b) Fluorescence measurements:*— Fluorescence emission was recorded on spectrofluorometer. Fluorescence of tyrosine residues of native (control) and peroxynitrite-modified histones were monitored in the wavelength range of 280-400 nm after excitation at 275 nm. Loss in emission intensity was computed using the following formula;

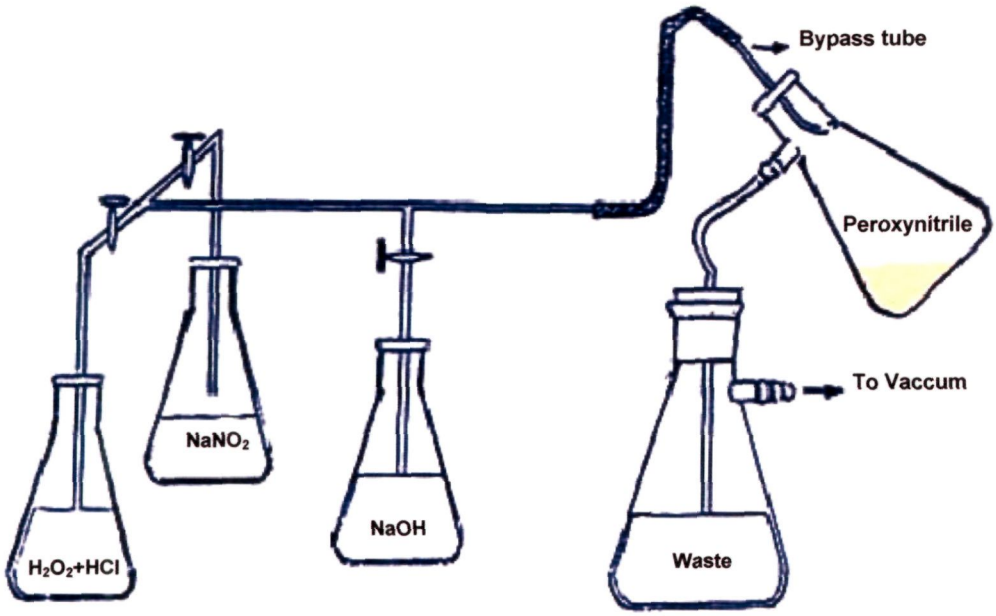



**Fig. 1 Assembly for laboratory synthesis of peroxynitrite.** The system was operated under vacuum and the bypass tube was initially directed to the waste bottle. Once a steady flow was observed the tube was manually twisted to collect the peroxynitrite. Barring waste container, all flasks were covered with ice.

$$\text{Percent loss in emission} = \frac{\text{emission of control histone} - \text{emission of peroxynitrite modified histone}}{\text{emission of control histone}} \times 100$$

Furthermore, fluorescence properties of ANS were utilized to study hydrophobic patches in control and peroxynitrite-modified histones. The ANS and histone ratio was kept at 1:5 and emission spectra were recorded in the wavelength range of 400-600 nm after excitation at 380 nm. Increase in fluorescence intensity (FI) was calculated as follows;

$$\text{Percent increase in FI} = \frac{\text{FI of peroxynitrite modified histone} - \text{FI of control histone}}{\text{FI of peroxynitrite modified histone}} \times 100$$

*(c) Circular dichroism (CD) measurements:*— Far-UV CD profile of histones were recorded on Jasco Spectropolarimeter. The machine was calibrated with D-10-camphorsulfonic acid and measurements were carried out at 25°C using temperature controlled cell holder attached to a Neslab's RTE 110 water bath with temperature accuracy of ± 0.1°C. Control and modified histone solutions (20 μM) were placed in 1 mm pathlength cuvette and profile was recorded in the wavelength range of 200-250 nm. A scan speed of 20 nm/min and response time of 1 sec was chosen to record CD spectra. Four sets of each sample was studied under identical conditions to confirm reproducibility of the results. The MRE (mean residual ellipticity) expressed in deg $cm^2$ $mol^{-1}$ was calculated using formula MRE = CD/10 x n x l x Cp where CD is in millidegree, n is the number of amino acid residues (205 for H1; 129 for H2A; 125 for H2B; and 135 for H3), l is the path length of the cell and Cp is the mole fraction. Helical content was calculated from the MRE values at 222 nm (Chen, *et al.*, 1972) using the equation;

$$\text{Percent alpha helix} = \left(\frac{\text{MRE}_{222\text{nm}} - 2340}{30300}\right) \times 100$$

*(d) Fourier transformed-infra red (FT-IR) spectroscopy:*— Lyophilized samples of native and peroxynitrite-modified histones were analyzed on FT-IR spectrometer. Briefly, pellets were prepared by mixing lyophilized samples with potassium bromide (to absorb remaining moisture, if any) under 5-6 T pressure

(produced by carver press) in an attenuated total reflection (ATR) cell attached to a thermostat; then the spectra were recorded (Oberg and Fink, 1998).

**Quantitation of nitrotyrosine in pronase digested histones by HPLC**

Pronase digested samples of histones (control as well as those modified by different doses of peroxynitrite) were subjected to HPLC analysis for nitrotyrosine. The retention time of standard 3-nitrotyrosine was taken as reference for comparison purposes. Briefly, the pronase protease was first dialyzed against 0.1 M sodium acetate buffer, pH 7.2 and added to histone samples (12.5 μM) present in 20 mM Tris buffer (pH 7.4). The pronase: histone ratio was 1:5 (w/w). The assay tubes were then kept at 50°C for 16 hr to ensure complete hydrolysis of the protein. The hydrolyzed samples were then subjected to ultrafiltration through 0.42 μM Milex filter. The filtrate was diluted 1:2 with eluant buffer (0.5 M potassium phosphate, pH 3.0 containing 10% methanol). The material was then injected into C-18 reverse phase column (25 cm x 4.6 mm; Vydac, USA) and elution was carried out at a flow rate of 0.8 ml/min. The absorbance of eluting material was monitored at 274 nm (the $\lambda_{max}$ of standard 3-nitrotyrosine in eluant buffer).

Identification of nitrotyrosine peak was made on the basis of its matching retention time with standard 3-nitrotyrosine and spiking experiments. The absorbance of two independent series of known concentration of standard nitrotyrosine (dissolved in eluant buffer) was used as reference to determine the concentration of nitrotyrosine in samples ( Kaur and Halliwell, 1994).

**HPLC analysis of patients' sera for free nitrotyrosine**

Serum samples of healthy control, SLE and RA patients were first diluted 1:1 (v/v) with eluant buffer and filtered. The filtrate was injected into C-18 column. The remaining steps were same as described above.

**Determination of reactive carbonyls in peroxynitrite-modified histones**

Peroxynitrite mediated oxidation of proteins result in alteration in the side chain structure of certain amino acid residues duo to generation of 2,4-dinitrophenylhydrazine (DNPH) reactive carbonyls. Spectrophotometric assay using DNPH reagent can give a quantitative estimate of carbonyl groups in proteins. In our studies, protein carbonyls were assayed according to the method of

Levine *et al.*, (1994) with slight modifications. Briefly, native and peroxynitrite–modified histones were precipitated with 10% (v/v) ice-cold trichloroacetic acid (TCA). After 10 min incubation at 4°C, the samples were centrifuged at 11,000 x g for 3 min. The pellet thus obtained was re-suspended in 0.5 ml of 10 mM DNPH dissolved in 2M HCl. The assay tubes were then placed in a sample holder and continuously vortexed at room temperature for 1 hr and precipitated with 0.5 ml of 20% (v/v) TCA followed by centrifugation at 11,000 x g for 3 min. The pellet was washed with 1 ml of 1:1 mixture of ethanol–ethyl acetate (v/v) to get rid of any extra DNPH reagent. Samples were incubated for 10 min at room temperature and then centrifuged at 11,000 x g for 5 min. The supernatant was discarded and the pellet was washed twice with ethanol-ethyl acetate mixture. The protein pellet was finally suspended in 1 ml of 6 M guanidinium chloride dissolved in 2 mM potassium phosphate buffer (pH 2.3, adjusted with trifluoroacetic acid). The samples were incubated at 37°C for 15-30 min for complete dissolution of proteins. Samples having difficulties going into solution were briefly sonicated and incubated at an elevated temperature, up to 70°C. All samples were then centrifuged to remove any insoluble material from suspension. The concentration of DNPH was determined by measuring absorbance at 360 nm against guanidinium chloride (as blank) using the molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$. Histone concentration was determined in the samples from their absorbance at 276 nm and respective extinction coefficient. Finally, the carbonyl content in native and peroxynitrite-modified histones was expressed as nmole/mg of protein.

**Studies on inhibition of nitration**

Effect of certain enzymatic and non-enzymatic inhibitors of nitration were evaluated as per the procedure descrived elsewhere (Yoshie and Ohshima, 1997). Solutions of histones were pre-incubated with 10 μM each of dithiothreitol, cysteine, glutathione, mannitol, ascorbic acid, vitamine E & uric acid and 500 units each of catalase and SOD. After necessary incubation, peroxynitrite was added and effect of quenchers on nitration was evaluated with proper control.

**Absorption-temperature scan**

Thermal denaturation of native and peroxynitrite-modified histones (dissolved in PBS, pH 7.4) were monitored at 280 nm on spectrophotometer

attached with a temperature programmer and controller assembly (Alam *et al.*, 1993). Percent denaturation of individual sample was calculated using the following equation;

$$\text{Percent denaturation} = \frac{A_T - A_{30}}{A_{max} - A_{30}} X100$$

where, $A_T$ is absorbance at different temperatures; $A_{max}$ is final maximum absorbance upon completion of denaturation (95 °C); and $A_{30}$ is initial absorbance at 30 °C.

## Immunization of animals

Fresh rabbits (1-1.5 Kg) were separately challenged with 200 µg each of H2A histone and its peroxynitrite-modified counterparts. The immunogen was first complexed with yeast RNA (one-third of immunogen amount) and then emulsified with equal volume of Freund's complete adjuvant (first injection). Intramuscular injections were given at multiple sites (Bustin and Stollar, 1972). Subsequent injections of respective immunogens were given in Freund's incomplete adjuvant at weekly intervals. During the course of immunization each animal received a total of 1200 µg of immunogen. A similar course of immunization was followed with RNA alone (66.5 µg). One week after the last dose, marginal ear veins of immunized animals were punctured and blood was carefully collected. Pre-immune blood was collected from all animals. Sera were separated and decomplemented by heating at 56°C for 30 min.

### Isolation of IgG by Protein A-agrose column

IgG from human and animal sera were isolated by affinity chromatography on Protein A-agarose column. Serum (0.3 ml) diluted with equal volume of PBS (pH 7.4) was applied on top of Protein A-agarose column equilibrated with above buffer. The wash through was recycled 2-3 times and unbound material was removed by extensive washing with PBS. The bound IgG was eluted with 0.58 % acetic acid in 0.85 % sodium chloride (Goding, 1978) and collected in a tube containing 1.0 ml of 1.0 M Tris-HCl, pH 8.5. Three ml fractions were collected and IgG concentration was determined considering 1.4 $OD_{280}$ = 1.0 mg IgG/ml. The isolated IgG was dialyzed against PBS, pH 7.4, and stored at –20°C with

0.1 % sodium azide. Elusion profile of one each of human and rabbit serum IgG has been given in Fig. 2 (a) & (b).

## Immunological detection of antibodies

Pre-immune and immune sera were tested for antibodies against native and peroxynitrite-modified histone by enzyme linked immunosorbent assay (ELISA) and gel retardation assay. Furthermore, sera of SLE and RA patients were processed in a similar way.

**Reagents for ELISA:** Following reagents were used in ELISA;

**Protein coating buffer:** 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6

**Tris-buffered saline (TBS):** 10 mM Tris, 150 mM NaCl, pH 7.4

**Tris-buffered saline containing Tween 20 (TBS-T):** 20 mM Tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4 containing 500 µl Tween-20 per litre.

**Carbonate-bicarbonate buffer:** 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6 containing 2 mM magnesium chloride.

**Substrate:** 500 µg p-nitrophenyl phosphate per ml of above carbonate-bicarbonate buffer.

## ELISA procedure

ELISA was carried out on flat bottom polysorp modules (Ali and Alam, 2002). Briefly, polysorp microtitre wells were coated with one hundred microlitres of 10 µg/ml of native/peroxynitrite-modified H2A (dissolved in protein coating buffer) and incubated for 2 hr at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the wells served as control devoid of antigen only. The antigen-coated wells were emptied and washed thrice with TBS-T to remove unbound antigen. Unoccupied sites were blocked with 150 µl of 2 % non-fat dry milk (in TBS, pH 7.4) for 4-5 hr at 37°C and then wells were washed once. In direct binding ELISA, sera (1:100 diluted)/purified IgG were directly added into antigen coated wells and incubated for 2 hr at 37°C and overnight at 4°C. The wells were emptied and extensively washed with TBS-T. Anti-immunoglobulin G alkaline phosphatase conjugate (diluted as per manufacturer's instructions) was added to each well. The conjugate was incubated at 37°C for 2 hr and then washed four times with TBS-T and three times with distilled water. Para-nitrophenyl

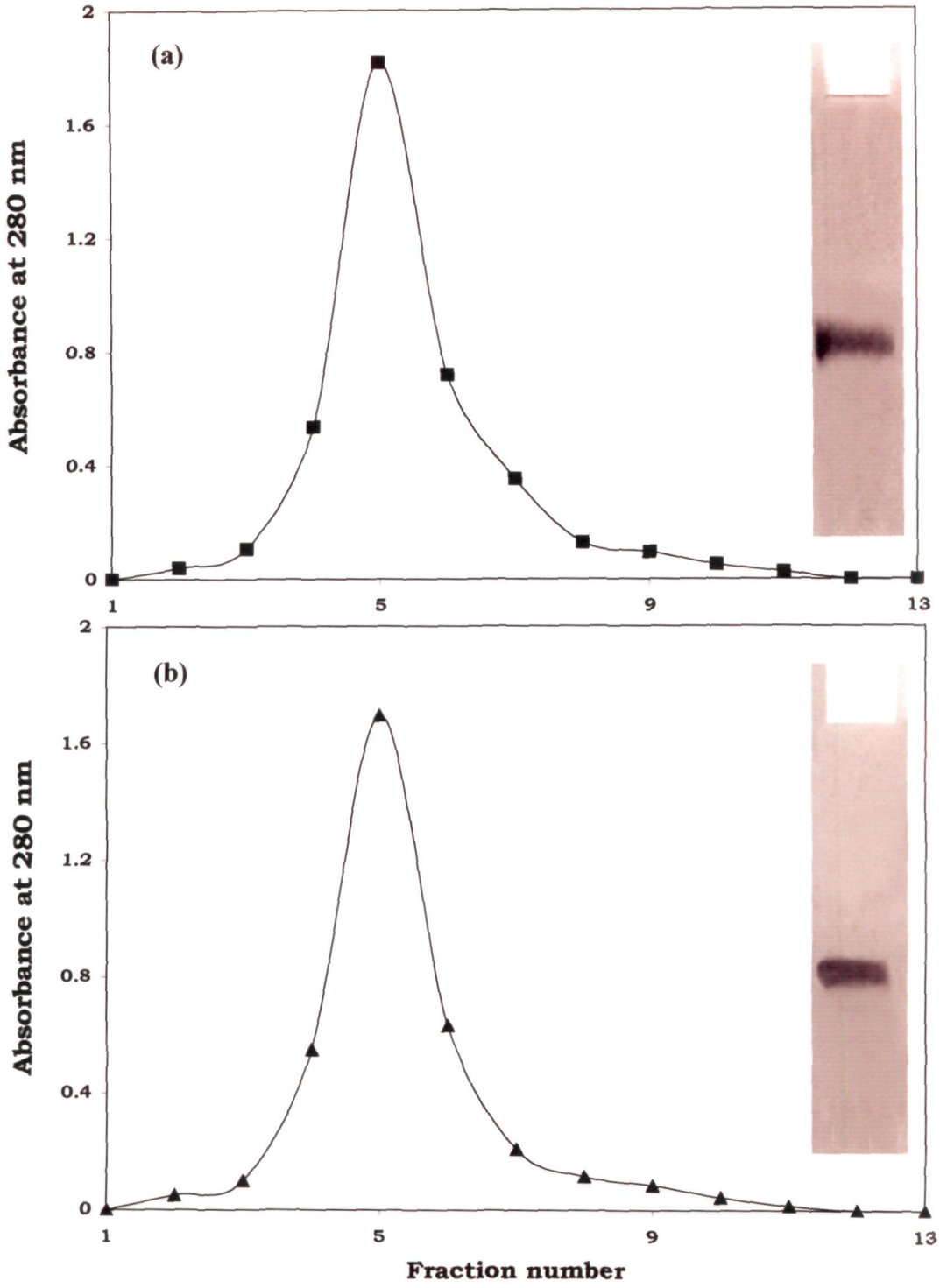



**Fig. 2** Elution profile of human serum IgG **(a)** and rabbit serum IgG **(b)** on Protein-A agarose column. **Inset:** Migration of purified IgG in 7.5% SDS-polyacrylamide gel.

phosphate was added and final color was read at 410 nm in a microplate reader. The results were expressed as mean of difference of absorbance values in test and control wells ($A_{test} - A_{control}$).

## Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA (Alam *et al.*, 2007). Varying amounts of inhibitors (0-20 µg/ml) were mixed with a constant amount of patient's sera/antisera or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum/IgG. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the following formula;

$$\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100$$

## Gel retardation assay

Antigen-antibody specificity was analyzed by mobility shift assay in polyacrylamide gel (Khan *et al.*, 2007). Immune complex was prepared by incubating constant amount of antigen (native and peroxynitrite-modified H2A) with varying amounts of anti-peroxynitrite-modified H2A antibodies for 2 hr at 37°C and overnight at 4°C. The reaction mixture was brought to room temperature and mixed with one-tenth volume of 0.002% bromophenol blue dissolved in a mixture of 0.5 M Tris (pH 6.8), 2% SDS and 10% glycerol. The samples were applied into the wells of 10% polyacrylamide gel and electrophoresed at 80 V for 3-4 hr in Tris-glysine buffer (pH 8.3) containing SDS. Bands were visualized by staining with silver nitrate.

## Statistical analysis

Data are presented as mean ± SD. Statistical significance of control versus test was computed using student's *t*–test (Statgraphics, Origin 6.1). A *p* value of 0.05 or less was considered statistically significant.

# Results

Purity of the commercially available calf thymus histones (H1, H2A, H2B and H3) was confirmed by single homogenous band movement in 10% SDS-polyacrylamide gel (figure not shown).

## Characterization of peroxynitrite

Peroxynitrite synthesized by rapid quenched flow method was identified by its $\lambda_{max}$ at 302 nm (Fig. 3) and characteristic yellow color. The concentration of peroxynitrite was conveniently assayed by diluting the stock into 1.2 M sodium hydroxide followed by absorbance measurement at 302 nm using molar extinction coefficient of 1670 $M^{-1}cm^{-1}$.

## Use of chemical quenchers to demonstrate generation of peroxynitrite

Since peroxynitrite is both nitrating and oxidizing agent, its *in vitro* generation was further confirmed by quenchers of nitration and oxidation or universal quenchers. The absorbance at 420 nm given by peroxynitrite–modified H2A histone was taken as control and effect of various quenchers was evaluated in comparison to above control. Uric acid, ascorbic acid, cysteine, dithiothreitol (DTT) and vitamin E caused decrease in nitration to the extent of 73, 65, 37, 30 and 50% respectively. Glutathione, SOD and catalase prevented the nitration to the extent of 43, 15 and 20% respectively. Mannitol, a hydroxyl radical scavenger, decreased the nitration to the extent of 10% only (Fig. 4).

## Absorption spectroscopy of native and peroxynitrite-modified histones

Pilot experiments were carried out to work out the optimum concentration of peroxynitrite needed to modify different histones. Histones (25 μM each) were incubated with varying concentration of peroxynitrite (50, 100 and 200 μM) and subjected to spectrophotometric analysis. As shown in Fig. 5, H1 histone gave absorbance peak at 276 nm. Upon modification with peroxynitrite the peak at 276 nm kept rising with increasing concentrations of peroxynitrite. Compared to 276 nm absorbance of H1 histone, the hyperchromicities shown by 50, 100 and 200 μM peroxynitrite-modified H1 histone was 26.3%, 42.8% and 60.0% respectively. Although the hyperchromicity shown by 200 μM peroxynitrite–modified H1 was maximum but the peak sharpness at 276 nm has almost vanished;

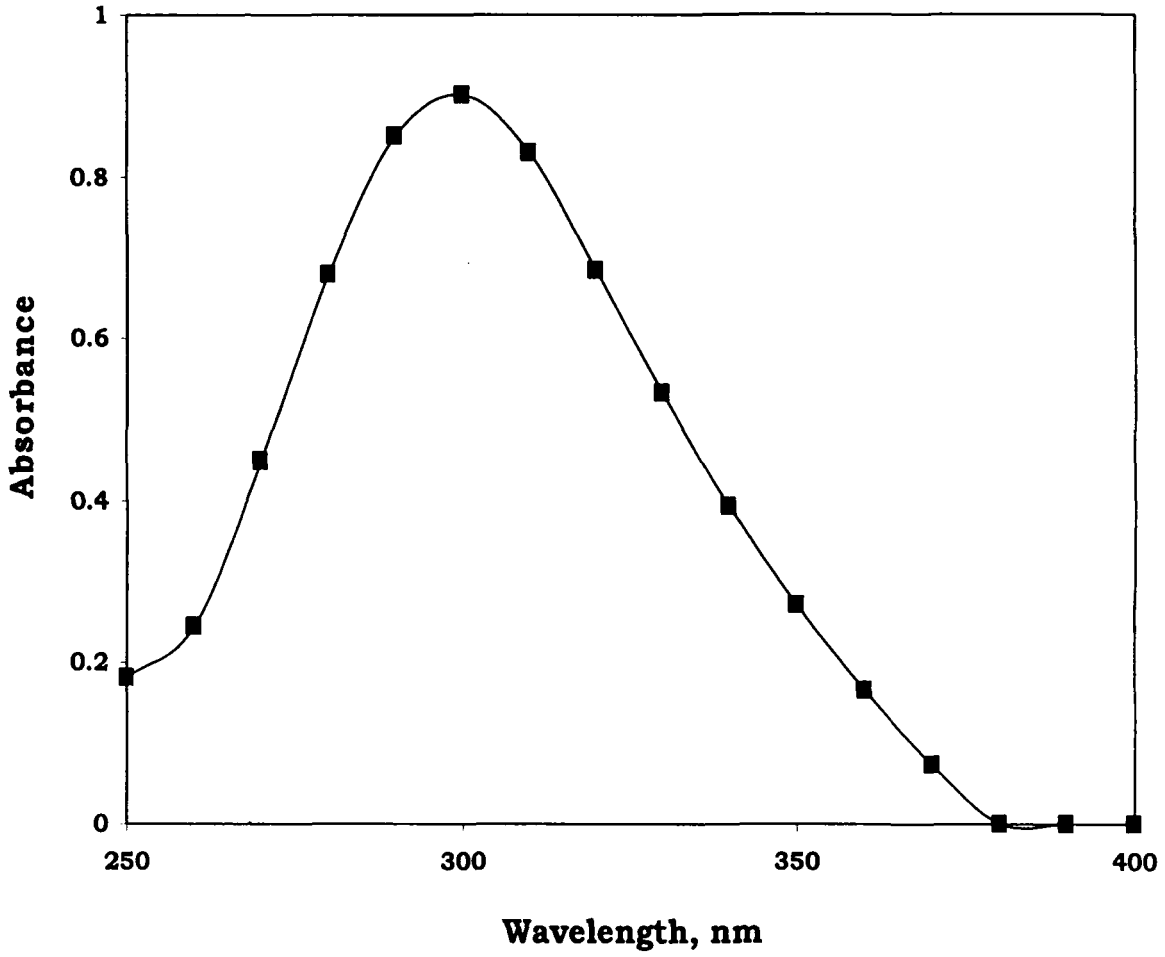



**Fig. 3** Absorption profile of peroxynitrite synthesized by quenched flow method.

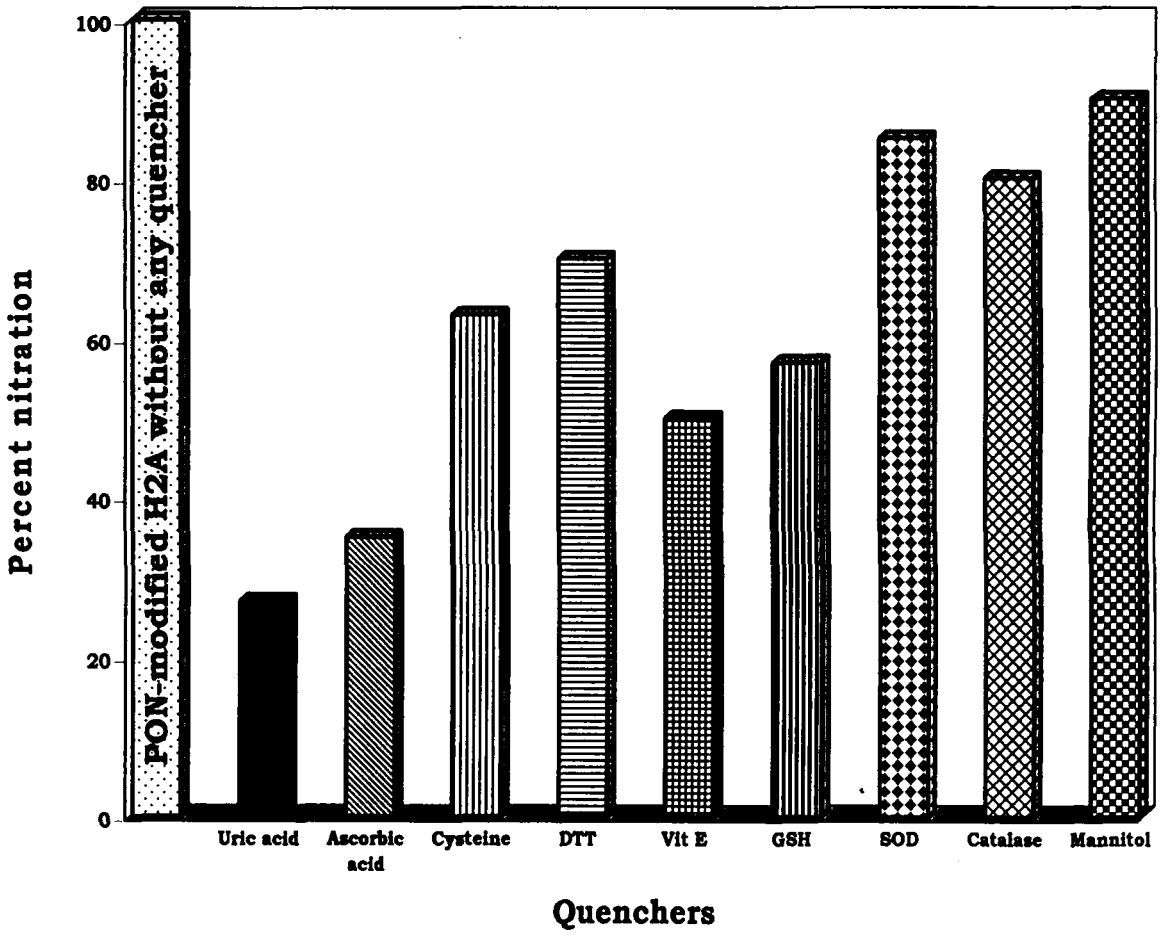



Fig. 4 Effect of some enzymatic and non-enzymatic quenchers on nitration of H2A by peroxynitrite.

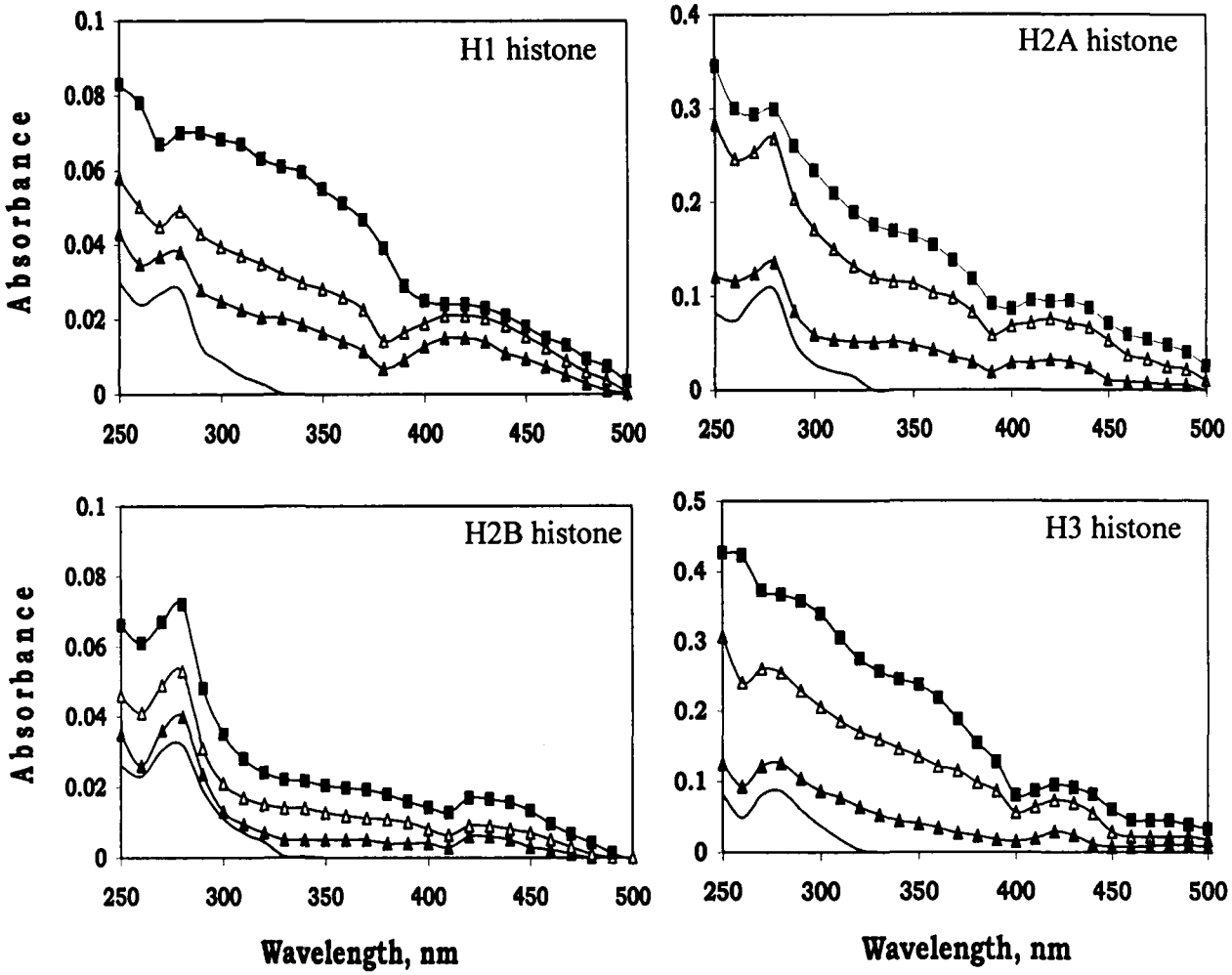



**Fig. 5** Absorbance profile of native histones (——) modified by 50 μM (—▲—), 100 μM (—Δ—) and 200 μM (—■—) peroxynitrite.

a characteristic which could be attributed to severe damage to structure responsible for otherwise typical sharpness at 276 nm. Furthermore, the peroxynitrite–modified counterparts of H1 histone gave birth to a new broad peak centered at 420 nm. This new peak was found to be due to generation of nitrotyrosine because a standard solution of 3-nitrotyrosine processed under identical conditions yielded similar absorbance profile (Fig. 6).

Under identical conditions and at equimolar concentration, H2A histone also gave maximum absorbance at 276 nm. Treatment of H2A histone with 50, 100 and 200 μM peroxynitrite resulted in hyperchromicity of 276 nm peak to the extent of 28.5%, 60% and 66% respectively. Furthermore, for peroxynitrite-H2A it may be noted that the sharpness of the peak was almost maintained even at the highest concentration of peroxynitrite; an indication that the overall structure of amino acid residues responsible for the typical absorbance of H2A at 276 nm is maintained. The broader but typical peak at 420 nm is a quasi evidence of progressive nitration of tyrosine residues in H2A with changing concentration of peroxynitrite. A close analysis of H2B histone and its peroxynitrite counterparts showed similar pattern of characteristic 276 nm $\lambda_{max}$ and hyperchromicities. However, modification of H2B histone with 50 μM peroxynitrite showed minimum hyperchromicity but the sharpness of 276 nm peak was almost maintained at irrespective of concentrations of peroxynitrite. The birth of 420 nm peak in H2B after peroxynitrite treatment may be attributed to increasing nitration of tyrosine residues in H2B histone. The absorption profile of H3 histone and its modified analogs are given in Fig. 5. In this case too we observed that the pattern of absorption of peroxynitrite-modified H3 analogs at 276 nm and 420 nm were similar. The 276 nm absorbance and percent hyperchromicity values of different histones and their peroxynitrite-modified counterparts are summarized in Table 3. The hyperchromicities seen in different histones modified with peroxynitrite is suggestive of structural perturbation in histone protein.

## Quantitation of nitrotyrosine in peroxynitrite-modified histone by HPLC

Figure 7 and 8 show HPLC profile of 3-ntrotyrosine taken as standard. We observed well defined peaks with a retention time of ~12.25 min. The peaks'

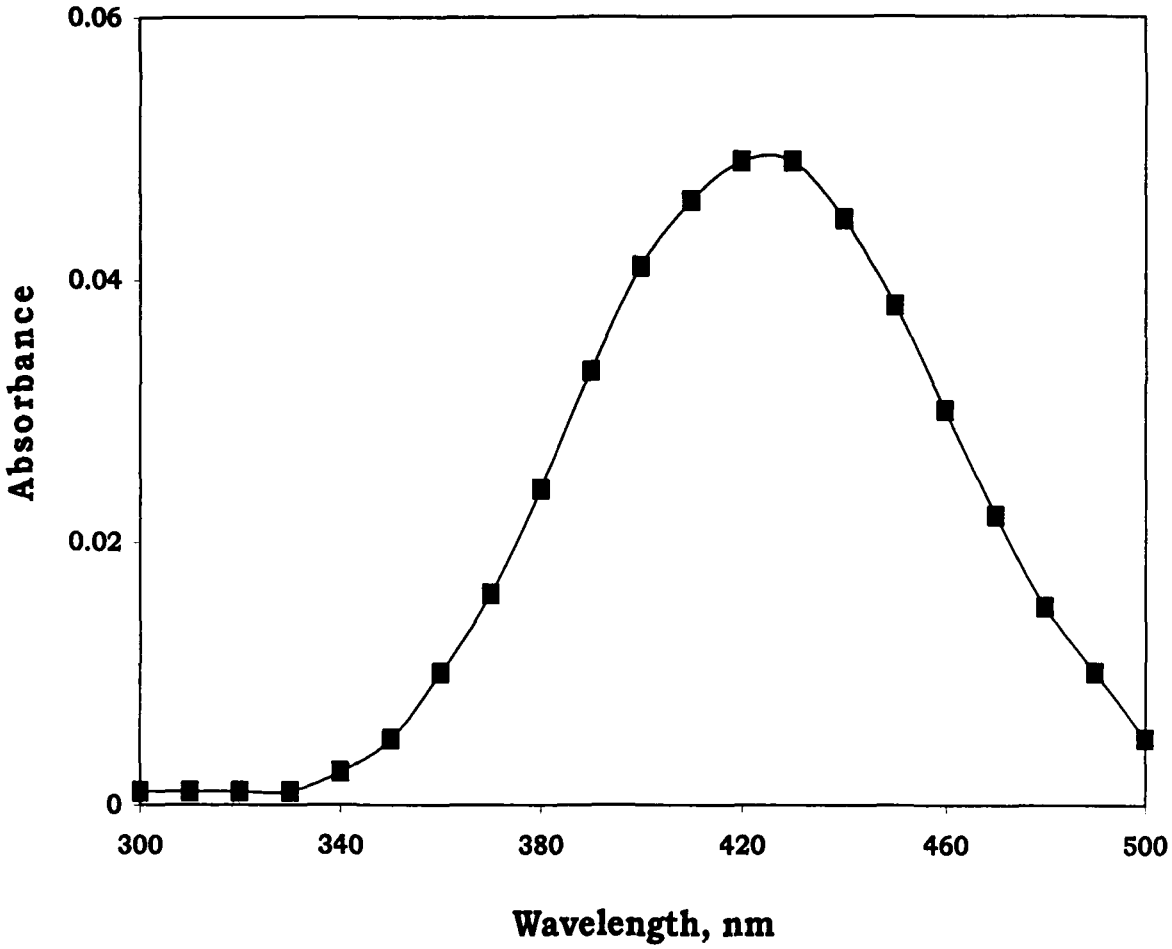



**Fig. 6** Spectrum of standard 3–nitrotyrosine.

## Table 3

**Absorption data on histones and their peroxynitrite–modified counterparts**

| Histone type | UV absorption at 276 nm | Percent hyperchromicity |
|---|---|---|
| $H1^N$ | **0.028** | — |
| | 0.038[a] | 26.3 |
| | 0.049[b] | 42.8 |
| | 0.070[c] | 60.0 |
| $H2A^N$ | **0.100** | — |
| | 0.107[a] | 21.3 |
| | 0.268[b] | 60.2 |
| | 0.290[c] | 64.2 |
| $H2B^N$ | **0.032** | — |
| | 0.040[a] | 20.0 |
| | 0.053[b] | 39.0 |
| | 0.070[c] | 54.0 |
| $H3^N$ | **0.086** | — |
| | 0.126[a] | 31.7 |
| | 0.225[b] | 66.2 |
| | 0.367[c] | 76.5 |

N = Native histone. **a, b** and **c** represent corresponding histones treated with 50 μM, 100 μM and 200 μM peroxynitrite.

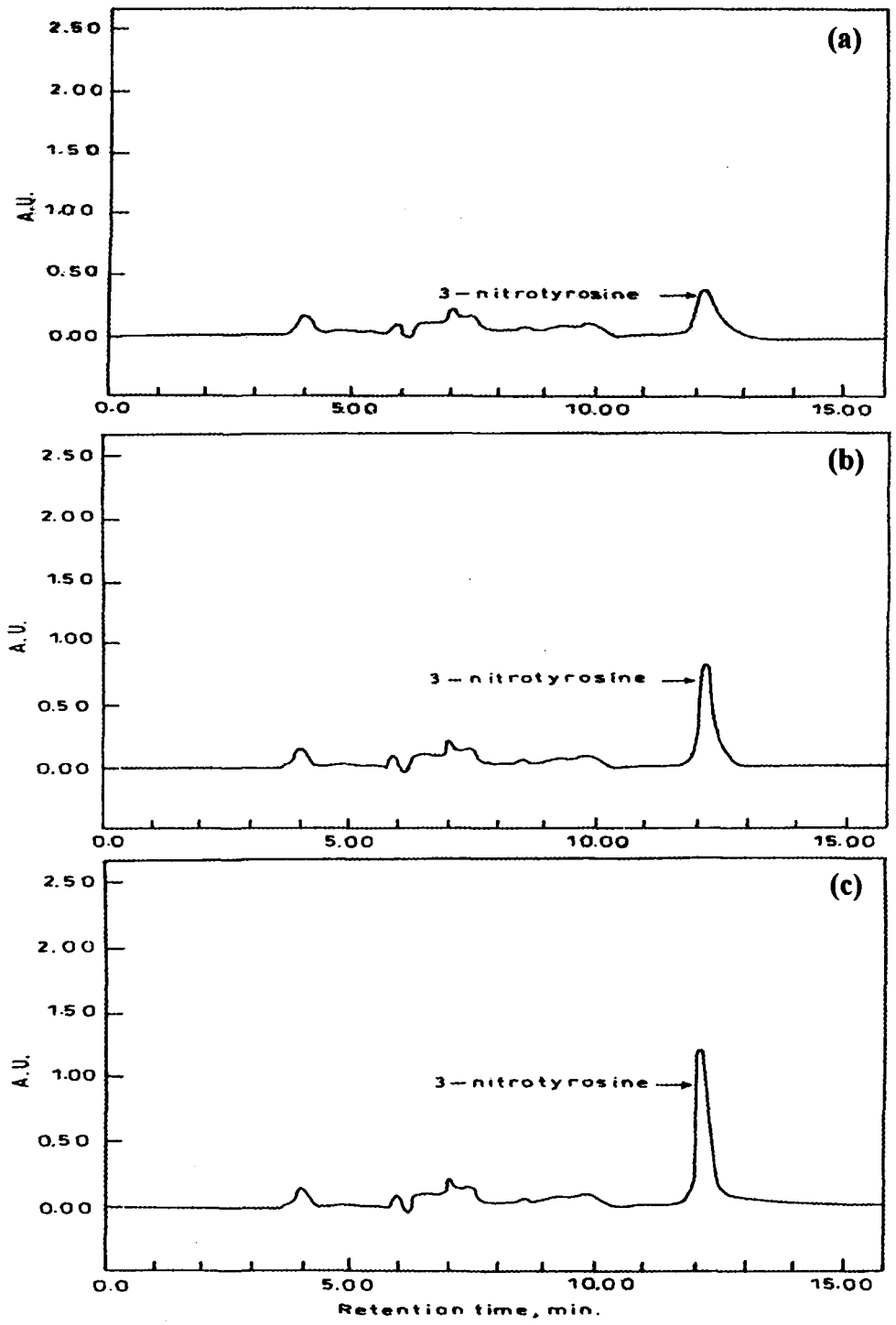



**Fig. 7** HPLC chromatogram of **(a)** 0.5 μM, **(b)** 1.0 μM, and **(c)** 2.0 μM standard 3-nitrotyrosine.

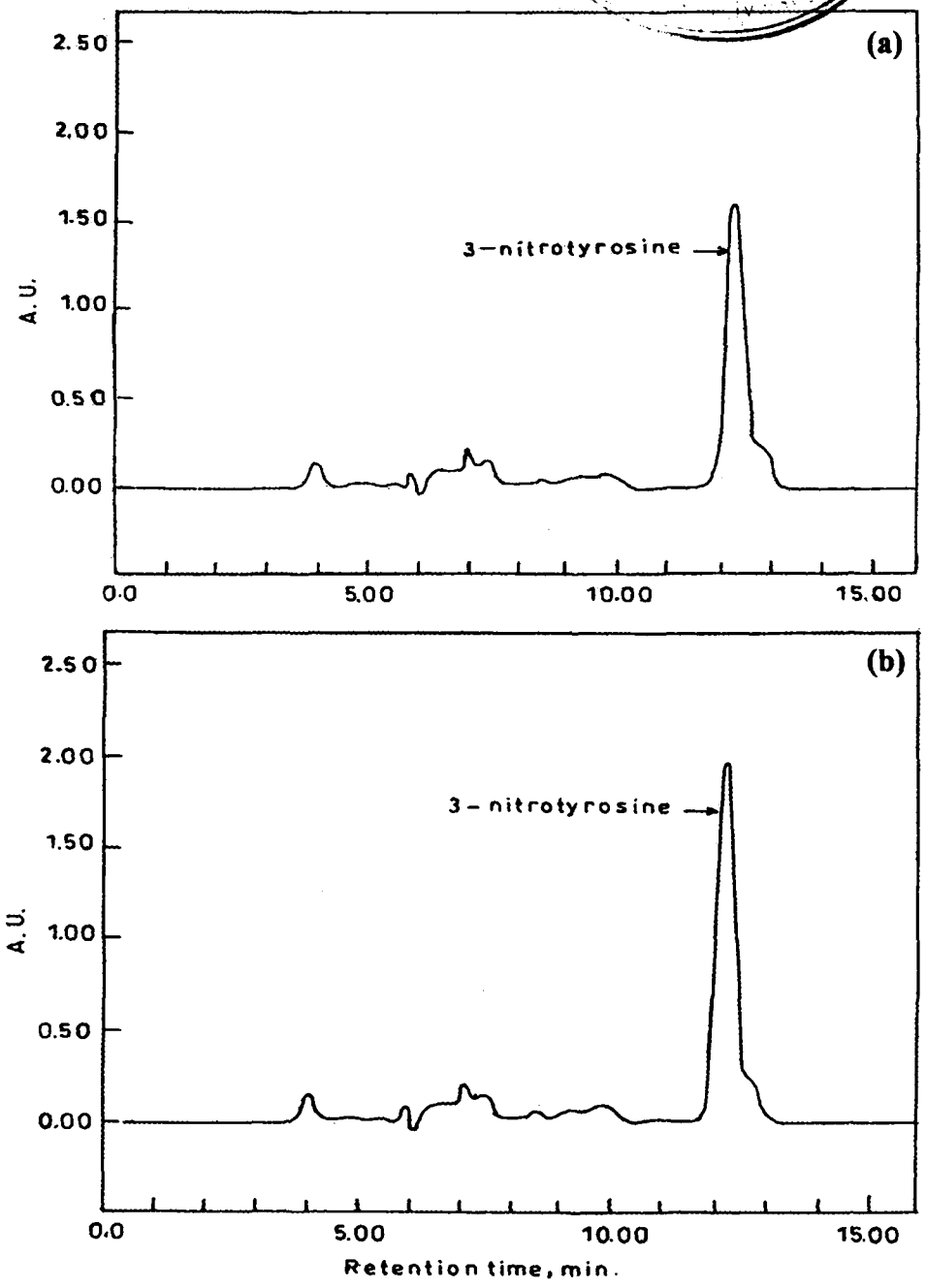



**Fig. 8** HPLC chromatogram of **(a)** 4.0 μM, and **(b)** 8.0 μM standard 3-nitrotyrosine.

absorbance was directly proportional to 3-nitrotyrosine concentration as depicted in Fig. 9. Analysis of 50 μM & 100 μM peroxynitrite–modified H1, H2A, H2B and H3 revealed generation of nitrotyrosine identified as well defined peaks with a retention time of ~12.5 min (Figs. 10-13). This matches with the retention time of approximately ~12.25 min shown by standard 3-nitrotyrosine. HPLC of pronase hydrolyzed native histones under identical conditions did not show any peak with retention time of ~12.5 min (Fig. 10**a**, 11**a**, 12**a** & 13**a**). It is concluded that the presence of nitrotyrosine in modified histones is the result of nitration caused by peroxynitrite. The nitrotyrosine content of the modified histones as calculated from the calibration curve of standard 3-nitrotyrosine (Fig. 9) has been summarized in Table 4.

## Fluorescence spectroscopy of native and modified-histones

Tyrosine contributes significantly to intrinsic fluorescence of proteins. As per the primary structure, there is one tyrosine in H1, three in each of H2A and H3 and five in H2B histone. The fluorophoric property of tyrosine residue was utilized to record the emission profiles of native histones and their peroxynitrite–variants after excitation at 280 nm. Emission profile of different histones and their peroxynitrite-counterparts are presented in Fig. 14. Irrespective of histone type, the fluorescence intensity was maximum at an emission wavelength of 305 nm; a characteristic associated with tyrosine. Even the peroxynitrite modification of these histones did not alter the wavelength of maximum emission. However, the emission intensity kept decreasing with increasing concentrations of peroxynitrite which may be attributed to nitration of tyrosine residues in corresponding histones. Table 5 depicts emission data on histones in the environment of peroxynitrite.

## ANS (1-anilinonaphthalene-8-sulfonic acid) fluorescence

Effect of peroxynitrite modification on the hydrophobic clusters in native and peroxynitrite–modified histones were probed with ANS. Fig. 15 shows ANS fluorescence spectra of peroxynitrite–modified H1, H2A and H2B and H3 histones respectively. It appears that the peroxynitrite-modification of above histones has gradually exposed the hydrophobic clusters/patches which are available for ANS-binding as indicated by increase in ANS fluorescence intensities and shift in the

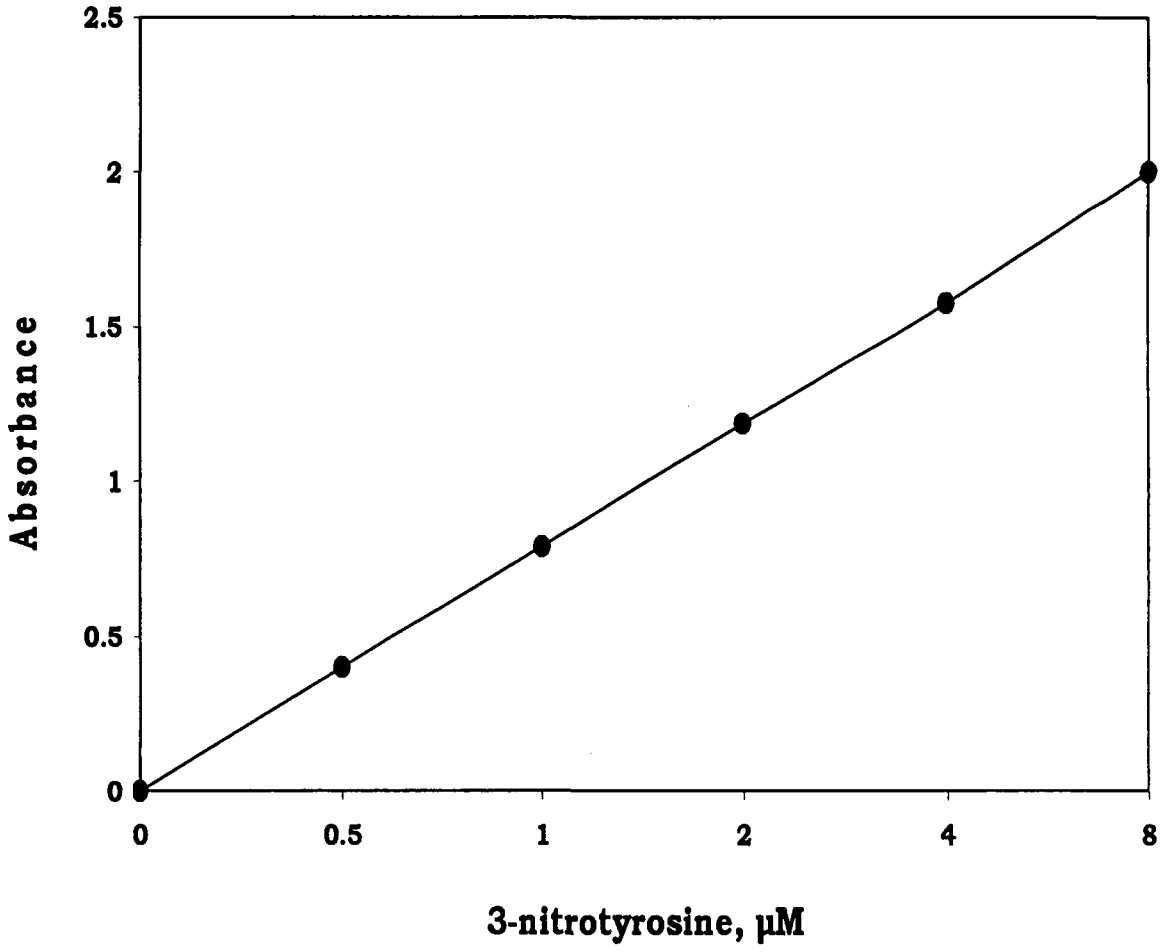



Fig. 9 Standard curve of 3-nitrotyrosine constructed from data in fig. 7 and 8.

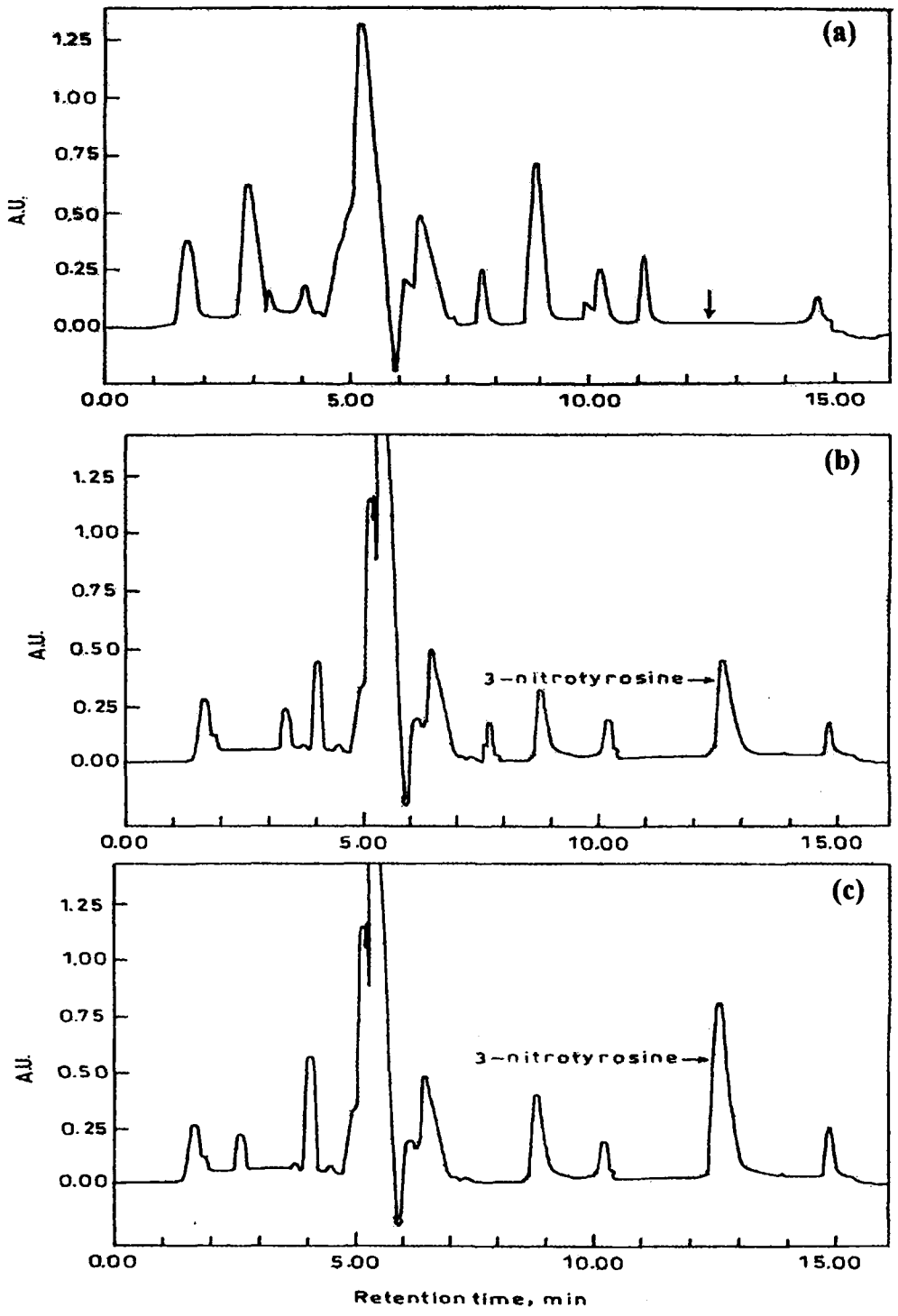



**Fig. 10** HPLC analysis of pronase treated histone. **(a)** native H1, **(b)** & **(c)** shows H1 histone modified with 50 μM and 100 μM peroxynitrite respectively.

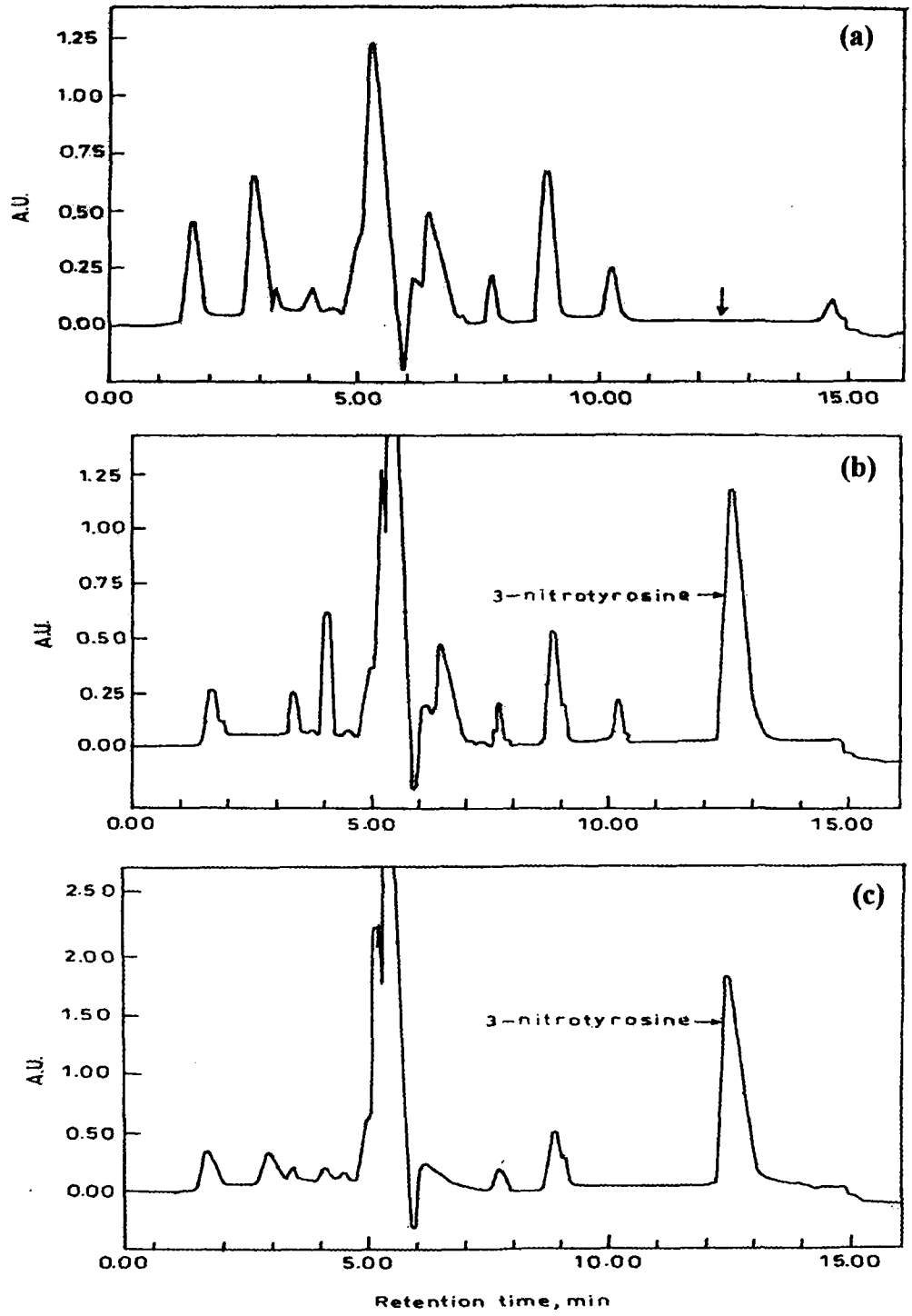



**Fig. 11** HPLC analysis of pronase treated histone. **(a)** native H2A, **(b)** & **(c)** shows H2A histone modified with 50 μM and 100 μM peroxynitrite respectively.

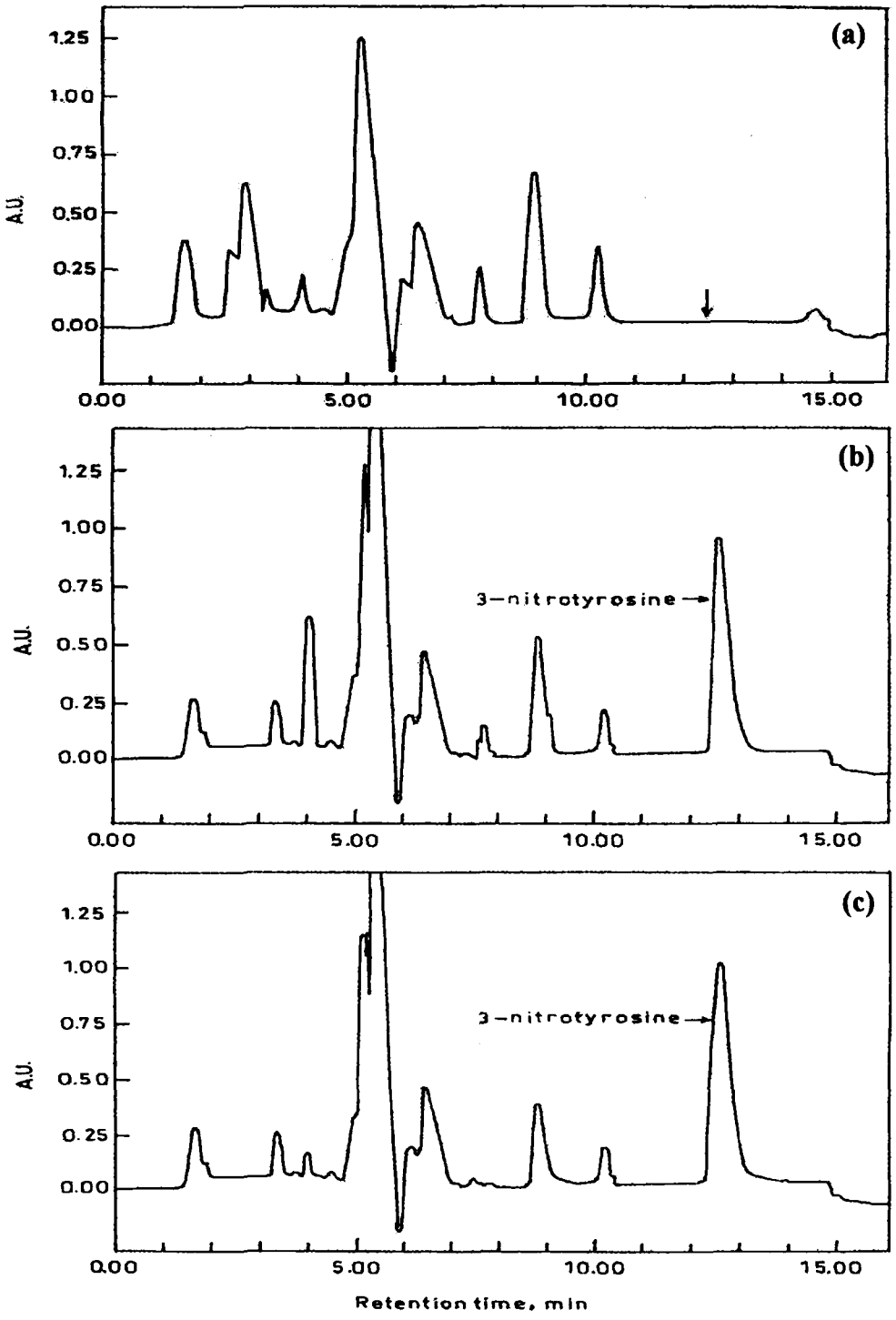



Fig. 12 HPLC analysis of pronase treated histone. **(a)** native H2B, **(b)** & **(c)** shows H2B histone modified with 50 μM and 100 μM peroxynitrite respectively.

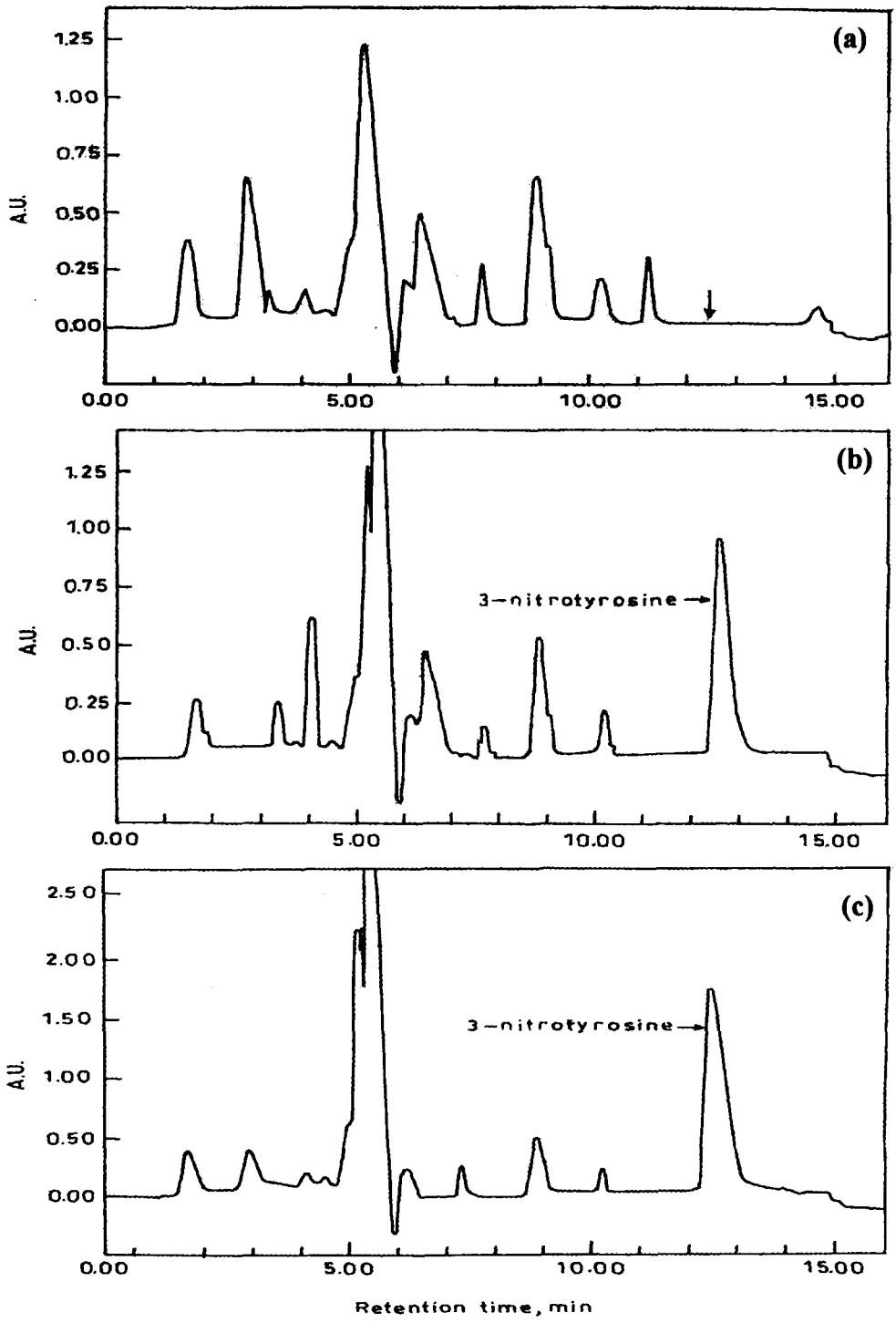



**Fig. 13** HPLC analysis of pronase treated histone. **(a)** native H3, **(b)** & **(c)** shows H3 histone modified with 50 μM and 100 μM peroxynitrite respectively.

## Table 4

**HPLC analysis of 3-nitrotyrosine in native and peroxynitrite–modified histones**

| Histone type | μM of nitrotyrosine |
|---|---|
| $H1^N$ | — |
| | 0.58[a] |
| | 1.00[b] |
| $H2A^N$ | — |
| | 1.83[a] |
| | 5.91[b] |
| $H2B^N$ | — |
| | 0.87[a] |
| | 1.63[b] |
| $H3^N$ | — |
| | 1.35[a] |
| | 5.04[b] |

N = Native histone. **a** and **b** represent corresponding histone treated with 50 μM and 100 μM peroxynitrite.

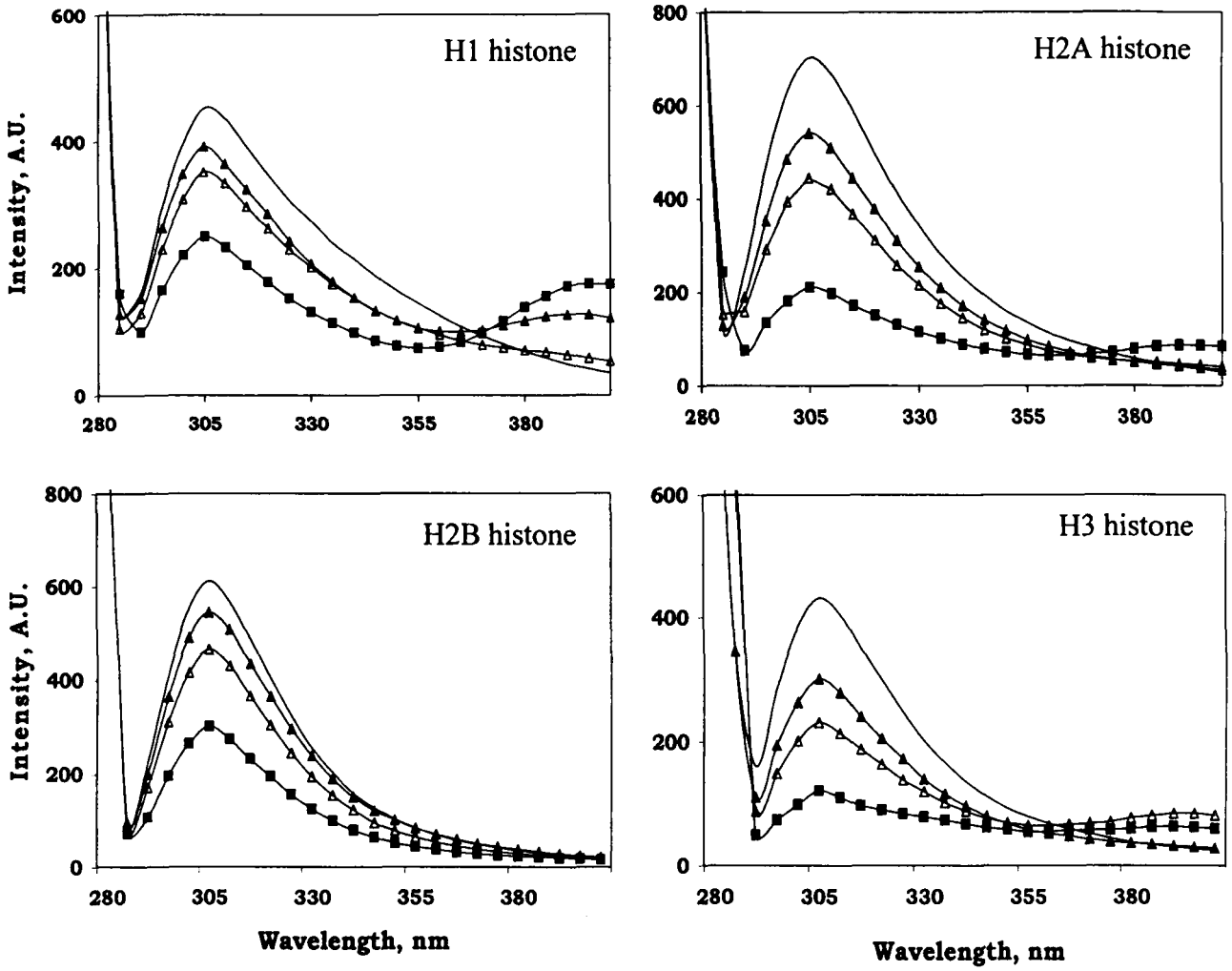



**Fig. 14** Fluorescence emission profile of native histones (——) modified with 50 μM (—▲—), 100 μM (—Δ—) and 200 μM (—■—) peroxynitrite.

## Table 5

**Fluorescence characteristics of native histones and their modified counterparts**

| Histone type | Intensity (A.U.) | Percent loss in intensity after modification |
|---|---|---|
| $H1^N$ | **453** | — |
| | 393[a] | 13.2 |
| | 353[b] | 22.3 |
| | 341[c] | 24.7 |
| $H2A^N$ | **706** | — |
| | 543[a] | 23.0 |
| | 446[b] | 36.8 |
| | 214[c] | 69.6 |
| $H2B^N$ | **612** | — |
| | 545[a] | 10.9 |
| | 461[b] | 24.6 |
| | 361[c] | 41.0 |
| $H3^N$ | **431** | — |
| | 274[a] | 36.4 |
| | 234[b] | 45.7 |
| | 122[c] | 71.6 |

N = Native histone. **a, b** and **c** represent corresponding histones treated with 50 μM, 100 μM and 200 μM peroxynitrite.

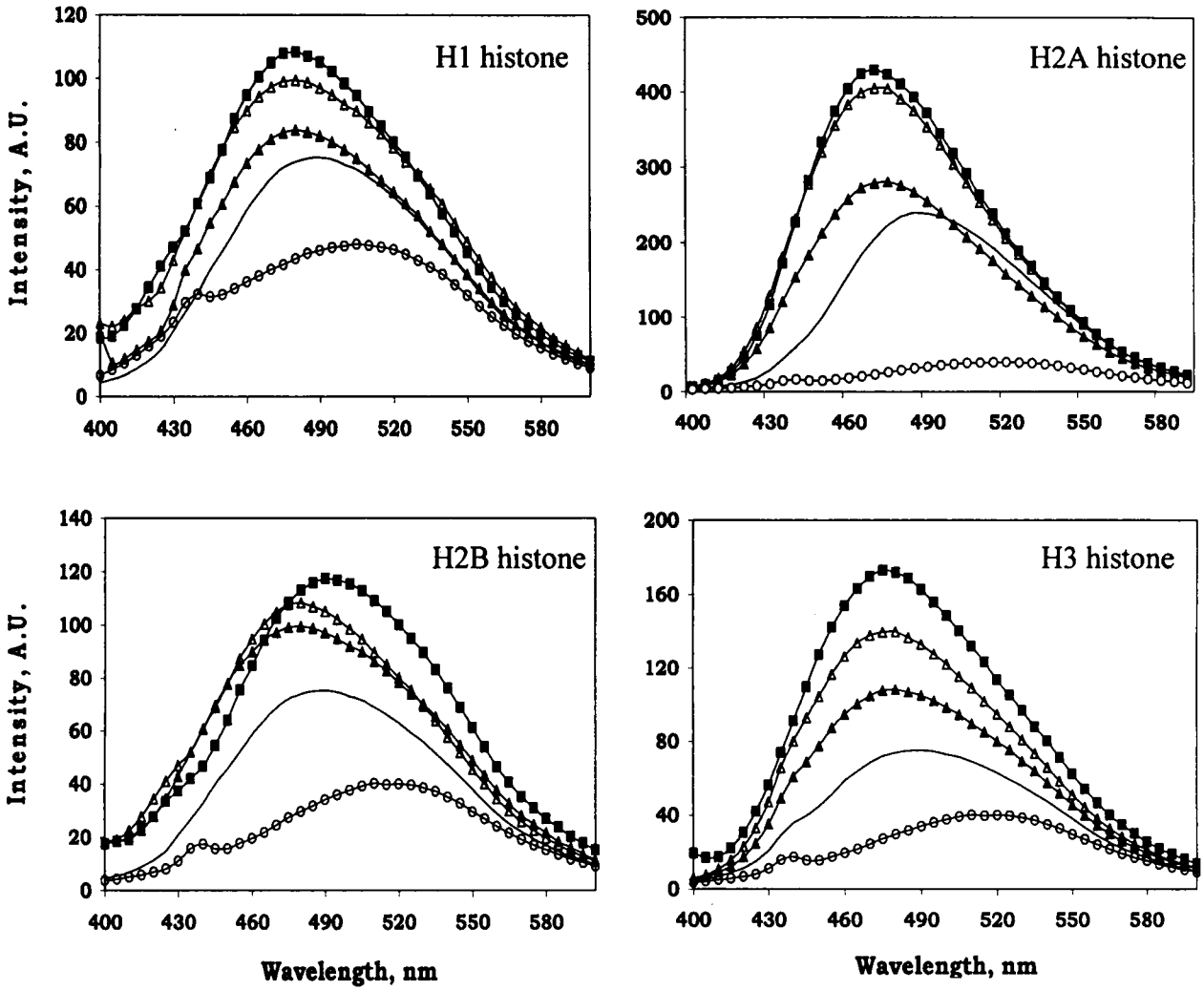



**Fig. 15** Fluorescence emission profile of ANS (—o—) binding to native histones (——) and the histones modified with 50 μM (—▲—), 100 μM (—Δ—) and 200 μM (—■—) peroxynitrite.

emission maximum wavelength. The results of ANS binding with native and modified histones are summarized in Table 6.

**SDS-polyacrylamide gel electrophoresis of histones**

Histones modified with peroxynitrite were subjected to non-reducing polyacrylamide gel electrophoresis under denaturing conditions. Twenty five μg each of native and peroxynitrite-modified histones were loaded into the wells of 10% polyacrylamide gels. The migration patterns of silver stained bands of native and peroxynitrite-modified histones are shown in Fig. 16 **A–D**. The native forms of all histones moved as compact single band. Moreover, H1, H2A and H3 histones modified with peroxynitrite (50 and 100 μM) showed retarded migration in the gel. Furthermore, it is quite clear that peroxynitrite-induced nitration and oxidation has resulted in cross linking of histones *vis-à-vis* formation of high molecular weight species. In case of H2B histone, the peroxynitrite-modified forms did not show retardation as observed for other histones under study. Instead, we observed a more prominent band in comparison to native H2B. It may be due to binding of more silver ions to additional aromatic amino acid residues available after treatment with peroxynitrite. During the study it was noticed that histones modified with 200 μM peroxynitrite did not enter the gel and clear cut bands were seen at the top of the well (results not shown). It may be concluded that higher concentrations peroxynitrite produced large aggregates which failed to enter the gel.

**CD spectroscopy of histones**

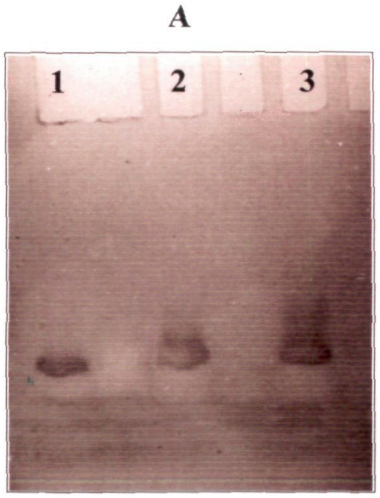
Circular dichroic (CD) studies provide rapid determination of proteins' secondary structure and thus a useful technique for quick assessment of conformation. The amide chromophores of the peptide bonds in proteins give CD reading in far-UV range (200-250 nm). Proteins rich in alpha-helix give negative bands near 222 nm and 208 nm. The circular dichroic profile of pure beta-sheets display negative bands near 216 nm and 175 nm and a positive band between 195-200 nm. The characteristic far-UV CD properties of proteins were exploited to look into the secondary structure of different histones and changes there in after modification with peroxynitrite. The far-UV CD profile of different histones is given in Fig. 17 & 18. The peroxynitrite-modified forms of histones showed changes in ellipticity at 222 nm and 208 nm compared to their non-modified

## Table 6

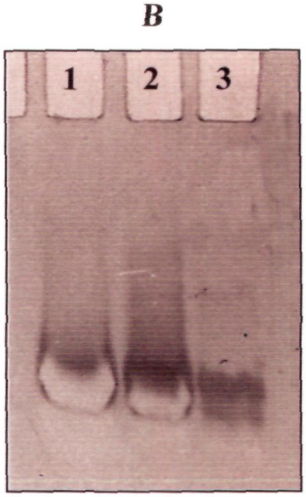
**ANS-binding characteristics of native and peroxynitrite–modified histones**

| Histone type | Fluorescence intensity (A.U.) | $\lambda_{max}$ (nm) | Shift in $\lambda_{max}$ (nm) | Percent increase in ANS binding to modified histones |
|---|---|---|---|---|
| $H1^N$ | **75.0** | **490** | — | — |
| | 84.0[a] | 483 | 07.0 | 10.7 |
| | 100.0[b] | 481 | 09.0 | 25.0 |
| | 108.0[c] | 480 | 10.0 | 30.5 |
| $H2A^N$ | **239.0** | **490** | — | — |
| | 281.0[a] | 472 | 18.0 | 15.0 |
| | 407.0[b] | 468 | 22.0 | 41.0 |
| | 430.0[c] | 468 | 22.0 | 45.0 |
| $H2B^N$ | **75.0** | **490** | — | — |
| | 100.0[a] | 482 | 08.0 | 25.0 |
| | 108.0[b] | 480 | 10.0 | 31.5 |
| | 117.0[c] | 480 | 10.0 | 36.0 |
| $H3^N$ | **76.0** | **490** | — | — |
| | 108.0[a] | 480 | 10.0 | 29.0 |
| | 139.0[b] | 478 | 12.0 | 45.3 |
| | 173.0[c] | 475 | 15.0 | 56.0 |

N =Native histone. **a, b** and **c** represent corresponding histone treated with 50 μM, 100 μM and 200 μM peroxynitrite.




H1 histone (lane 1) modified with 50 & 100 μM peroxynitrite (lanes 2 & 3) respectively.




H2A histone (lane 3) modified with 50 & 100 μM peroxynitrite (lanes 2 & 1) respectively.


H2B histone (lane 3) modified with 50 & 100 μM peroxynitrite (lanes 2 & 1) respectively.


H3 histone (lane 1) modified with 50 & 100 μM peroxynitrite (lanes 2 & 3) respectively.

**Fig. 16** SDS-polyacrylamide gel (10%) of native histones and their peroxynitrite–modified variants. Electrophoresis was carried for 4 hr at 70 volts and the gels were stained with silver nitrate.

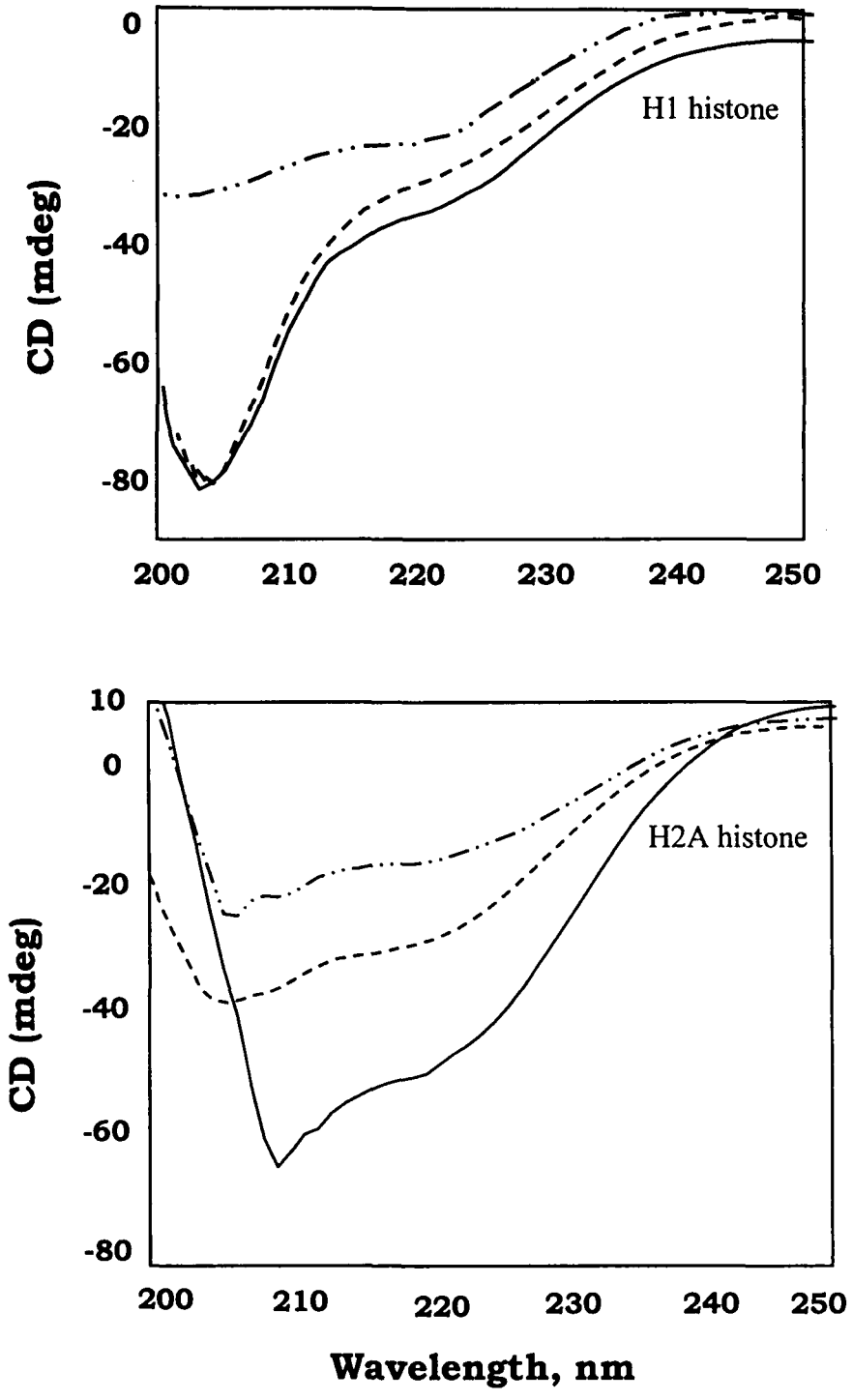



**Fig. 17** Far–UV CD spectra of native H1 and H2A histones (——) modified with 50 μM (-----) and 100 μM (— · · —) peroxynitrite.

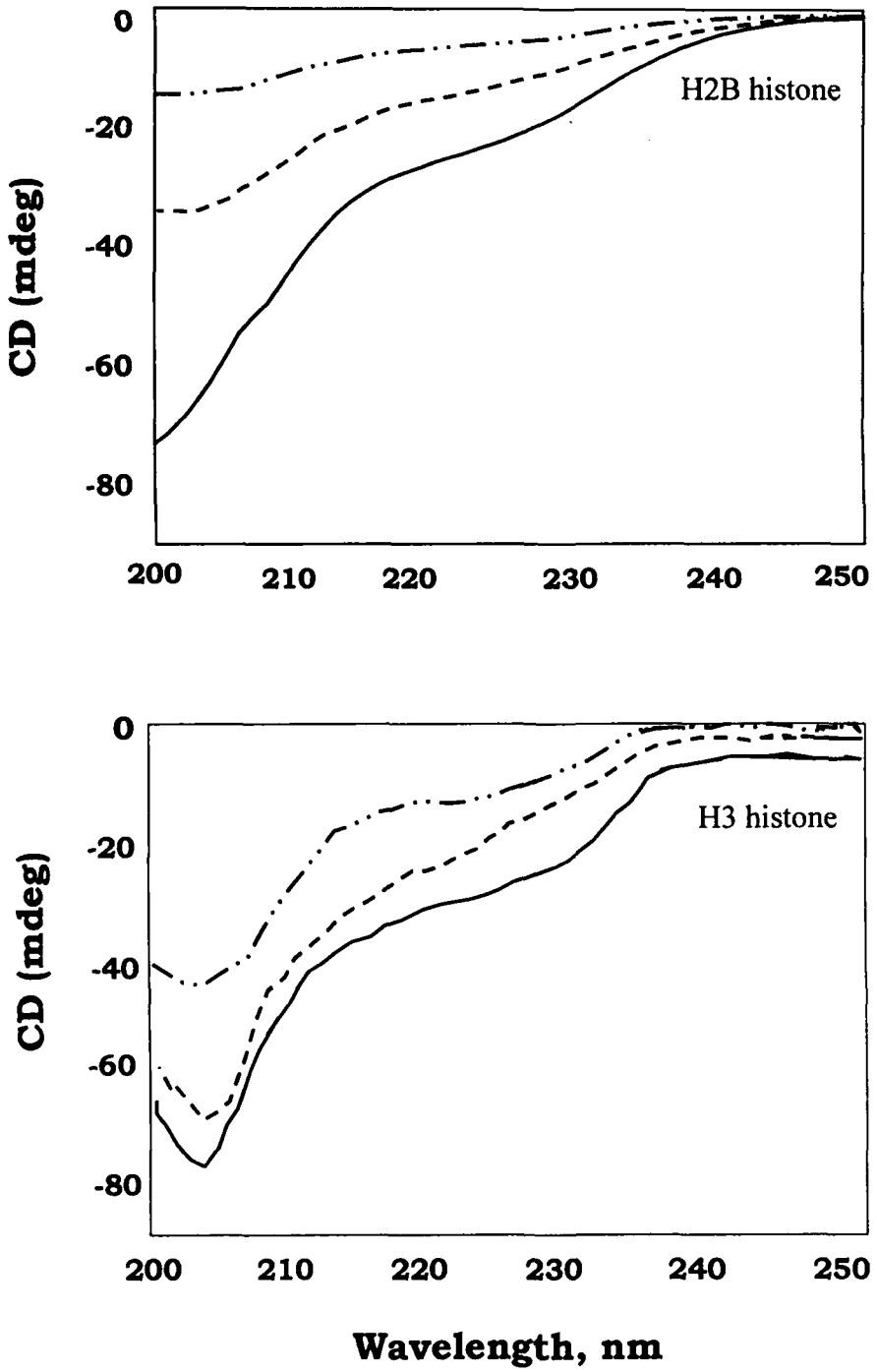



**Fig. 18** Far–UV CD spectra of native H2B and H3 histones (——) modified with 50 μM (-----) and 100 μM (— · · —) peroxynitrite.

counterparts. The pronounced negative ellipticities at 208 nm in case of non-modified histones (except H2B) are indicative of short alpha-helical regions. It appears that peroxynitrite modification of histones has destroyed alpha-helical content of these basic proteins as reflected by increase in ellipticity values of modified conformers. The MRE and alpha helical contents of different histones are summarized in Table 7.

**FT-IR analysis of native and 100 μM peroxynitrite-modified histones**

FT-IR is much more sensitive than CD spectroscopy for studying β-structure in proteins, although higher concentrations of sample are required in FT-IR experiments. Fig. 19 represents the FT-IR profile of native forms of H1, H2A, H2B and H3 histones at pH 7.5. In native forms, the maximum absorbance at a particular wavenumber is referred to as amide 1 region. The spectra of peroxynitrite–modified histones show significant difference compared to the native histones, indicating the structural changes. The most evident change was the appearance of a new band near the wavenumbers 1645 (PON–H1), 1625 (PON–H2A), 1640 (PON–H2B) and 1635 (PON–H3) respectively, which correspond to β-sheets. It means that, in contrast to native forms, the peroxynitrite–modified histones contain significant amount of β-structure under the given experimental conditions. FT-IR spectra permitted quantitative analysis of the secondary structure of histones. These results are summarized in Table 8. Analysis of data shows that histones modified with 100 μM peroxynitrite possess significant amount of β-structure compared to corresponding native histones.

**Quantitation of dityrosine in peroxynitrite-modified histones**

Peroxynitrite reaction with tyrosine residues in proteins generate phenoxyl radicals. These radicals then form C–C covalent bond (cross-link or dityrosine) between the aromatic moieties of phenolic tyrosine residues. The amount of dityrosine in peroxynitrite–modified histones was calculated. Dityrosine is characterized by broad peak of absorbance at 330 nm $\lambda_{max}$. The dityrosine content in histones modified with peroxynitrite (50, 100 and 200 μM) are summarized in Table 9.

## Table 7

**CD characteristics of native and peroxynitrite–modified histones**

| Histone type | MRE | Percent alpha-helix |
|---|---|---|
| $H1^N$ | **15853.66** | **44.6** |
| | 13414.64[a] | 36.5 |
| | 10365.85[b] | 26.5 |
| $H2A^N$ | **13333.33** | **36.3** |
| | 08205.13[a] | 19.4 |
| | 05128.21[b] | 09.2 |
| $H2B^N$ | **10047.62** | **25.4** |
| | 05952.38[a] | 10.0 |
| | 02658.73[b] | 01.1 |
| $H3^N$ | **11481.48** | **30.2** |
| | 08518.52[a] | 20.4 |
| | 05296.30[b] | 09.7 |

N = Native histone. **a** and **b** represent corresponding histones treated with 50 μM and 100 μM peroxynitrite.

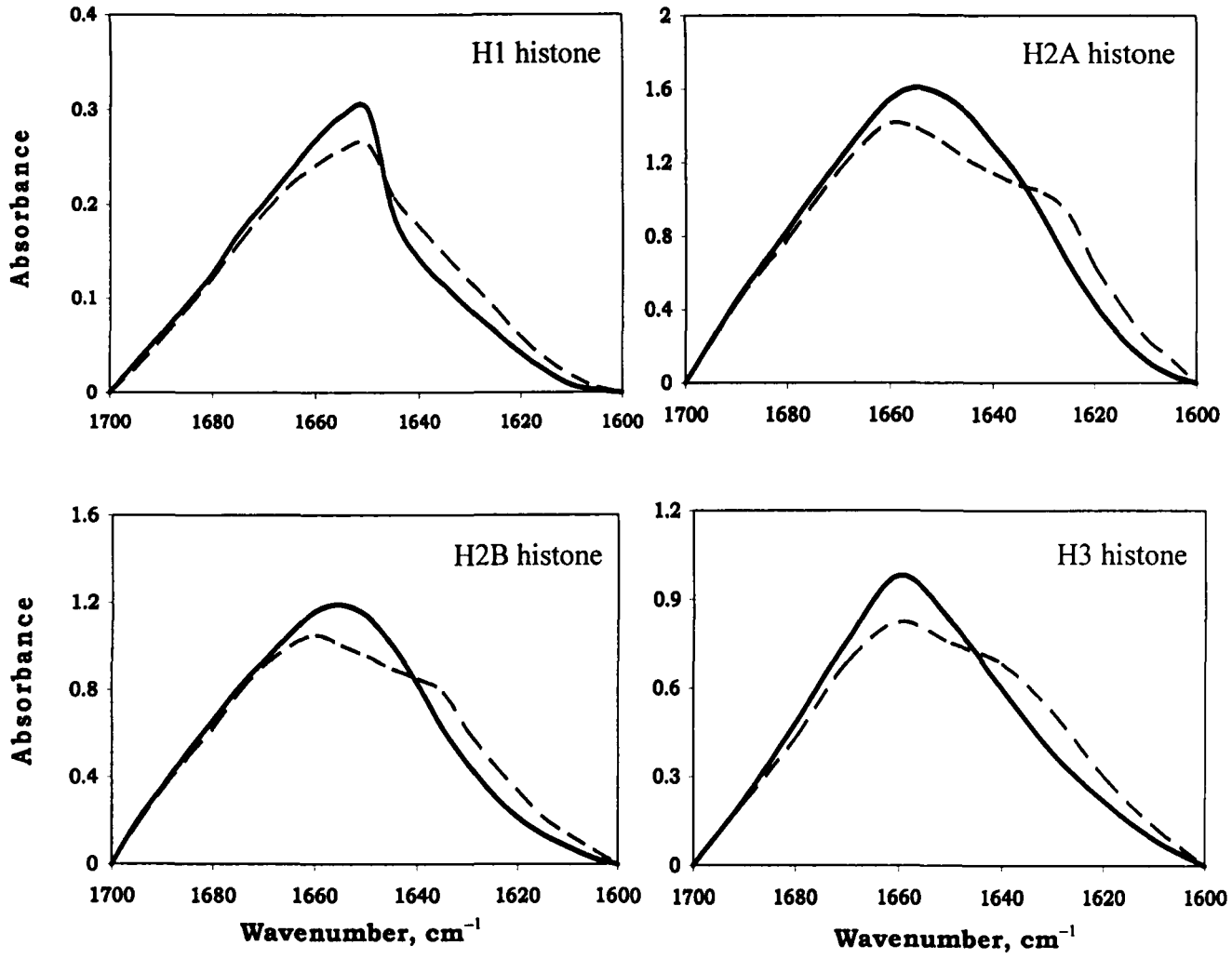



**Fig. 19** FTIR of native histones (——) and their corresponding counterparts modified with 100 μM peroxynitrite (– – – –).

## Table 8

**FT-IR data on native and peroxynitrite–modified histones**

| Histone | Wavenumber ($cm^{-1}$) | Percent modification loss at each native wavenumber ($cm^{-1}$) |
|---|---|---|
| $H1^N$ | **1650.63** | — |
| | 1645.17[a] | 12.30 |
| $H2A^N$ | **1658.43** | — |
| | 1625.31[a] | 13.62 |
| $H2B^N$ | **1656.76** | — |
| | 1635.12[a] | 15.60 |
| $H3^N$ | **1657.56** | — |
| | 1640.17[a] | 16.00 |

N = Native histone, and **a** represent corresponding histone treated with 100 μM peroxynitrite.

## Table 9

**Dityrosine content of histones modified with peroxynitrite**

| Histone type | μM of dityrosine |
|---|---|
| $H1^N$ | — |
| | 0.2[a] |
| | 3.5[b] |
| | 5.5[c] |
| $H2A^N$ | — |
| | 13.2[a] |
| | 40.0[b] |
| | 64.2[c] |
| $H2B^N$ | — |
| | 5.1[a] |
| | 8.0[b] |
| | 15.2[c] |
| $H3^N$ | — |
| | 12.7[a] |
| | 30.0[b] |
| | 44.0[c] |

N = Native histone. **a, b** and **c** represent corresponding histones treated with 50 μM, 100 μM and 200 μM peroxynitrite.

## Melting studies on native and peroxynitrite-modified histones

Effect of peroxynitrite-modification on the stability of different histones was studied by recording heat induced changes in absorbance values. The mid-point melting temperature (Tm) was calculated from different absorbance values at 280 nm in the temperature range of 30-90°C. The thermal denaturation profiles of native and peroxynitrite–modified histones are depicted in Fig. 20. We observed that treatment of histones with peroxynitrite produced thermal stability as shown by increase in the melting temperatures of peroxynitrite–modified samples of histones. Formation of cross links and/or disulphide bridge(s) appears to be the main reason for increased stability of peroxynitrite–modified histones. Table 10 summarizes the Tm values of native and modified samples.

## Carbonyl estimation in native and peroxynitrite-modified histones

Peroxynitrite–mediated protein oxidation may result in cleavage, cross-linking and modification of amino acid side chains. Carbonylation of lysine, arginine, threonine and proline residues are established markers of protein oxidation. The carbonyl groups are detected by their derivatization with 2,4-dinitrophenyl hydrazine. The reaction forms 2,4-dinitrophenyl hydrazone which is quite stable and measured spectrophotometrically at 360 nm.

The carbonyl content in native H1 histone was found to be 7.8 nmole/mg of protein. Upon modification with 50 μM, 100 μM and 200 μM peroxynitrite, the carbonyl content increased to 9.3, 13.7 and 19.0 nmole/mg of protein respectively (Table 11). The carbonyl was also estimated in other histones (both in native and modified conditions) and the data are summarized in the above table. The data simply points out that the formation of nitrotyrosine in histones is increasing with change in peroxynitrite concentration, but not without oxidation of the proteins.

## Immunogenicity of native and peroxynitrite-modified H2A histone

Since H2A histone was found to be maximally nitrated under our experimental conditions, we chose only this histone for antibody induction. Antisera of animals immunized with native and peroxynitrite–modified H2A histone were tested for antibody titre by direct binding immunoassay on polysorp wells coated with respective immunogens.

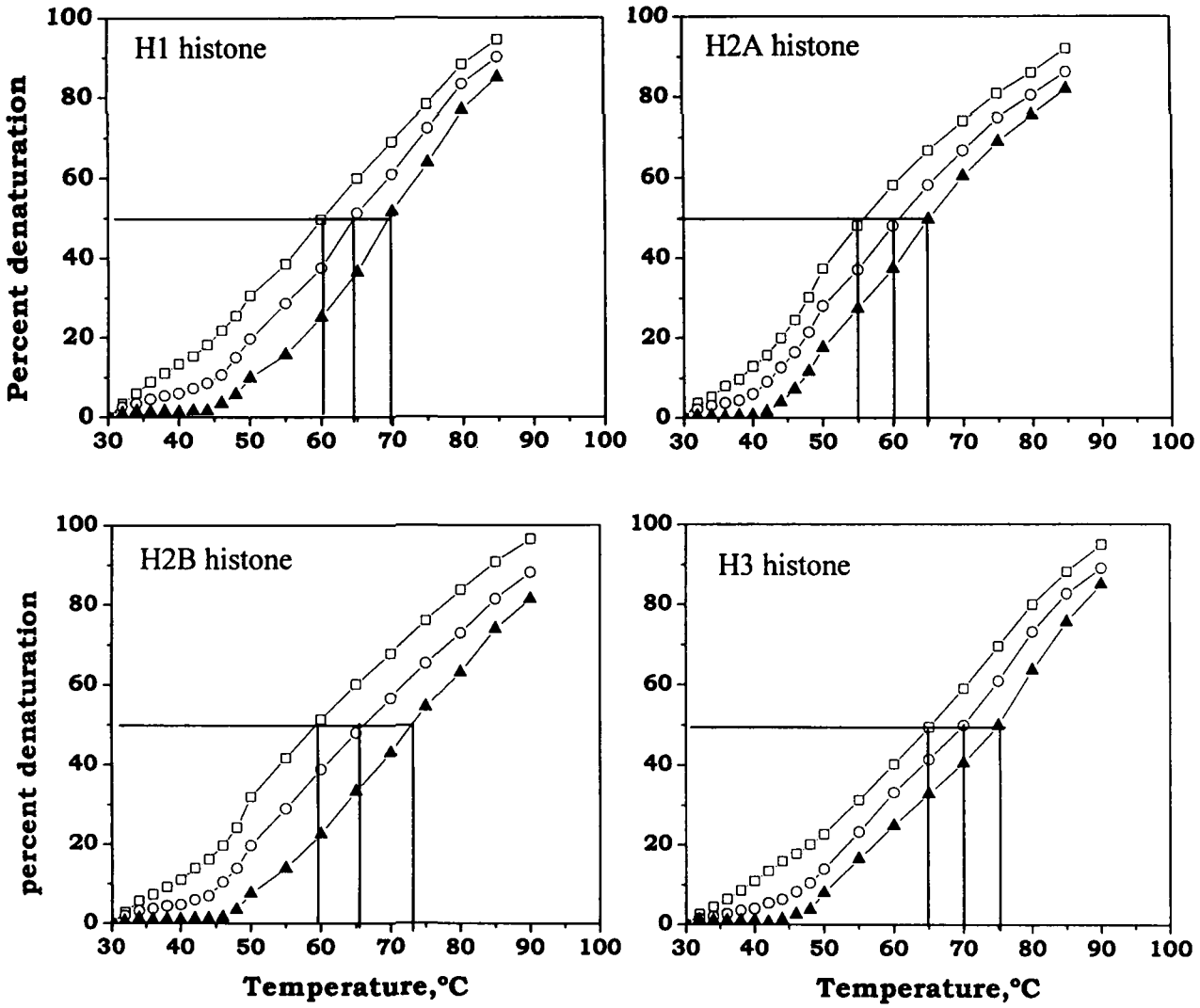



**Fig. 20** Thermal denaturation profile of native histones (—□—) modified with 50 μM (—○—) and 100 μM (—▲—) peroxynitrite. The samples were kept in PBS (pH 7.4) at a concentration of 10 μM.

## Table 10

**Melting temperature (Tm) of native and peroxynitrite–modified histones**

| Histone type | Tm, °C |
|---|---|
| $H1^N$ | **60.0** |
| | 64.1[a] |
| | 69.4[b] |
| $H2A^N$ | **55.0** |
| | 60.0[a] |
| | 64.4[b] |
| $H2B^N$ | **58.7** |
| | 65.9[a] |
| | 72.5[b] |
| $H3^N$ | **64.8** |
| | 70.0[a] |
| | 75.0[b] |

N = Native histone. **a** and **b** represent corresponding histones treated with 50 μM and 100 μM peroxynitrite.

# Table 11

**Carbonyl content of histones modified with peroxynitrite**

| Histone type | Carbonyl contents (nmole/mg protein) | Percent increase in carbonyl level compared to control |
|---|---|---|
| $H1^N$ | **7.8** | — |
| | 9.3[a] | 16.0 |
| | 13.7[b] | 43.0 |
| | 19.0[c] | 60.0 |
| $H2A^N$ | **6.0** | — |
| | 8.3[a] | 27.0 |
| | 10.0[b] | 40.0 |
| | 14.0[c] | 57.0 |
| $H2B^N$ | **6.9** | — |
| | 9.2[a] | 25.0 |
| | 14.8[b] | 53.0 |
| | 17.2[c] | 60.0 |
| $H3^N$ | **7.5** | — |
| | 10.0[a] | 25.0 |
| | 15.2[b] | 50.0 |
| | 21.0[c] | 64.0 |

N = Native histone. **a, b** and **c** represent corresponding histones treated with 50 μM, 100 μM and 200 μM peroxynitrite.

Antiserum against 50 μM peroxynitrite–modified H2A showed an antibody titre of 1:6400 (Fig. 21**c**). Modification with 100 μM peroxynitrite conferred more immnuogenicity on H2A histone and a titre of > 1:25600 was seen (Fig. 21**d**). Native H2A induced moderate antibody response (Fig. 21**a**). Yeast RNA used as carrier was non-immunogenic (Fig. 21**b**).

Specificity of induced antibodies against 50 μM and 100 μM peroxynitrite-modified H2A with corresponding immunogens is shown in Fig. 22 **a**, **b**. It was observed that the H2A histone modified with 100 μM peroxynitrite was not only a powerful immunogen but its antibodies are more specific. Since modification of H2A histone by 100 μM peroxynitrite induced best antibody response, further studies were carried out with this antigen and the corresponding antibodies induced against it.

**Purification of serum IgG**

Immunoglobulin G was isolated from preimmune and immune sera by affinity chromatography on Protein A-agarose column. Protein A (from *Staphylococcus aureus*) binds to IgG from most mammalian species through unique histidine residues present on Fc region of the molecule. Purity of IgG isolated from each of a rabbit– and human serum is shown by single band movement in SDS-polyacrylamide gel under non-reducing conditions (Fig. 2**a** & **b** and inset there in).

IgG purified from preimmune serum and antiserum against 100 μM peroxynitrite-modified-H2A histone were subjected to direct binding immunoassay on microtitre wells coated with 100 μM peroxynitrite-modified H2A histone. The IgG purified from immune serum showed strong binding with the coated antigen (Fig. 23). Preimmune IgG showed negligible binding under identitical conditions. The specificity of isolated anti-100 μM peroxynitrite-modified H2A IgG was evaluated by inhibition ELISA using immunogen as inhibitor and coating antigen (Fig. 24). The antibody specificity for the immunogen was evident from 93% inhibition. Comparison of data in Fig. 22 and Fig. 24 suggest that antigen-antibody interaction is more specific with purified IgG.

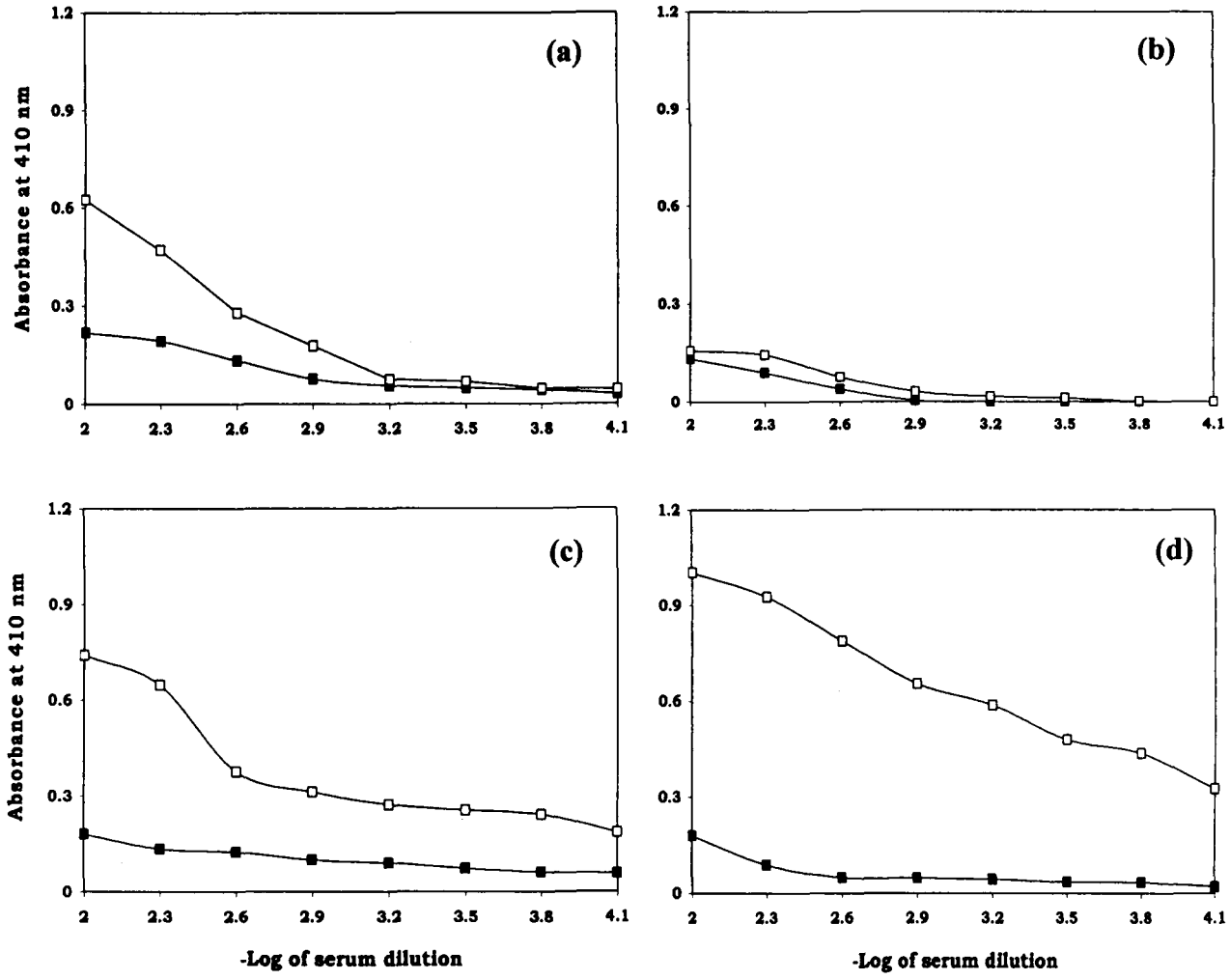



**Fig. 21** Direct binding ELISA of antiserum (—□—) against **(a)** native H2A, **(b)** yeast RNA **(c)** H2A modified with 50 μM peroxynitrite, and **(d)** H2A modified with 100 μM peroxynitrite. The microtitre wells were coated with respective immunogens. Preimmune serum binding to corresponding immunogen has been shown by filled squares (—■—).

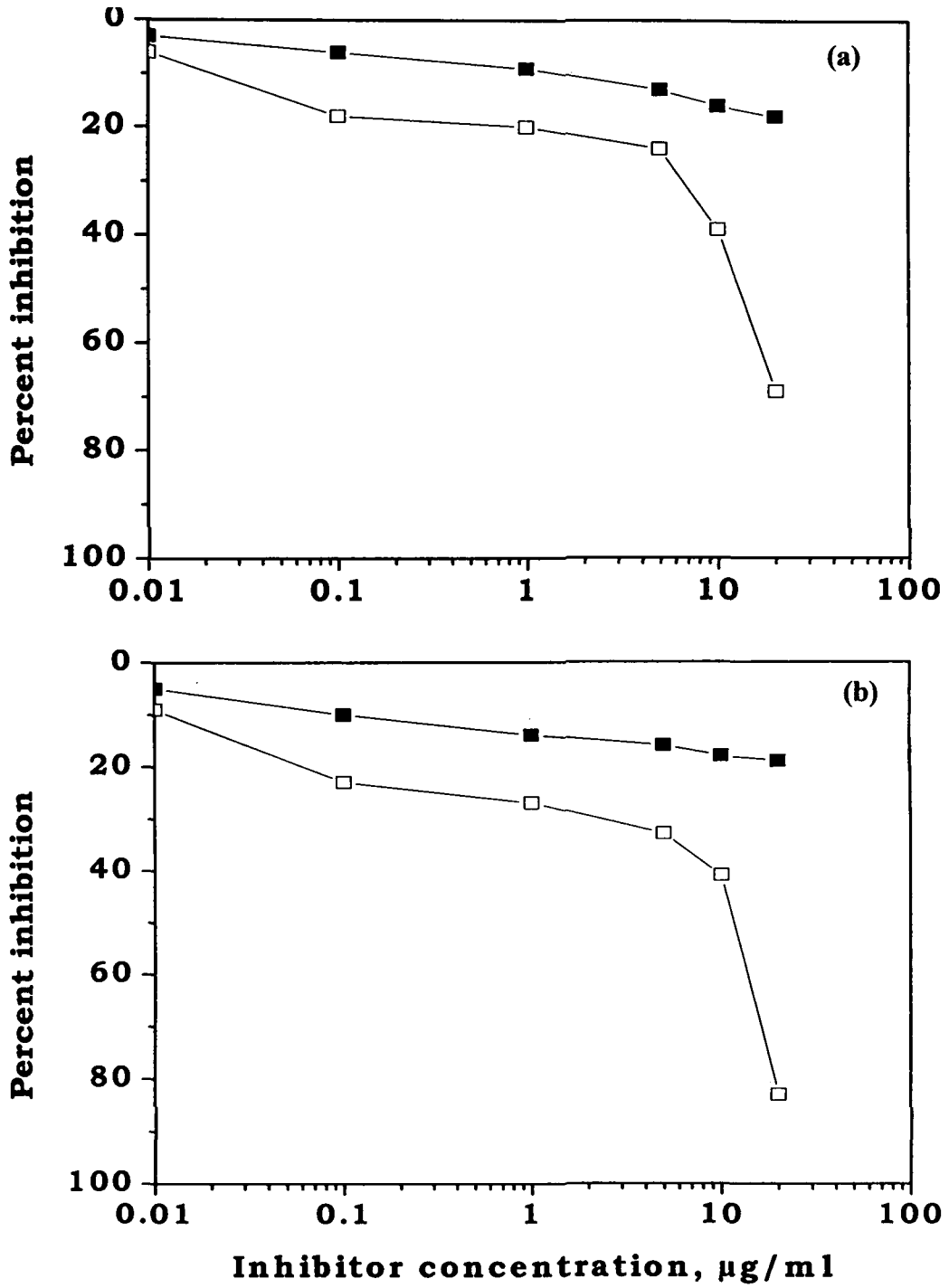



Fig. 22 Inhibition of binding of preimmune (—■—) and immune (—□—) serum antibodies by H2A modified by 50 μM peroxynitrite **(a)**, and H2A modified by 100 μM peroxynitrite **(b)**.

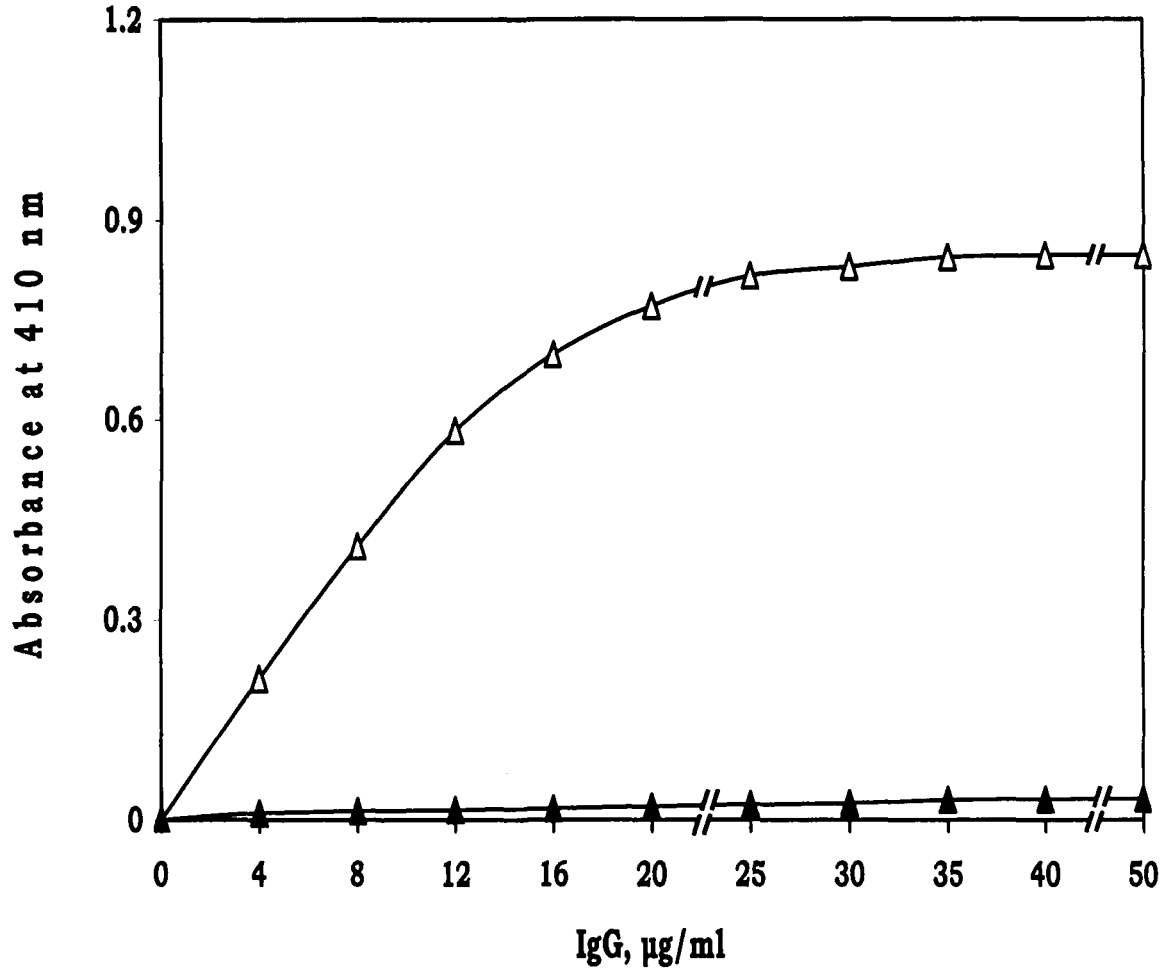



Fig. 23 Direct binding ELISA of affinity purified immune IgG (—Δ—) and preimmune IgG (—▲—). The microtitre wells were coated 100 µM peroxynitrite–modified H2A histone.

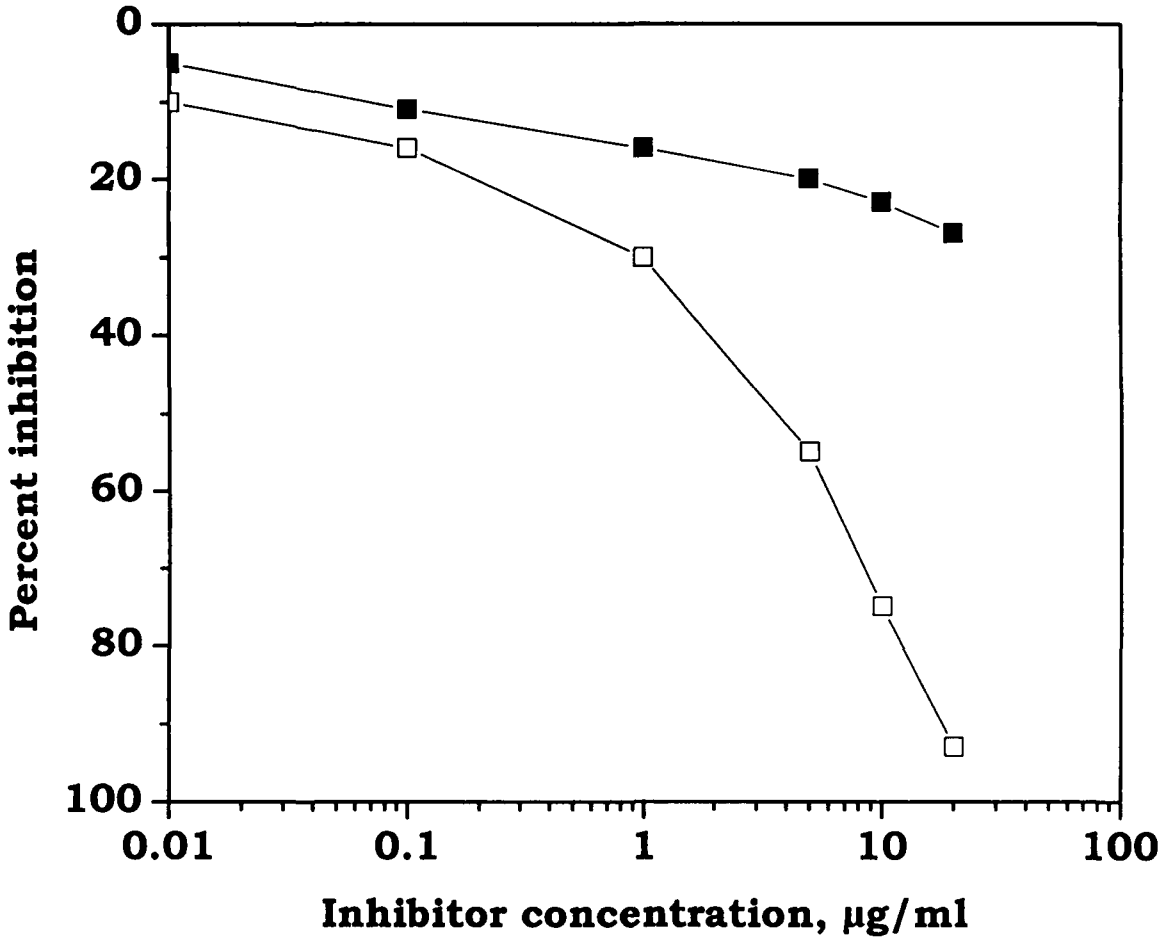



**Fig. 24** Inhibition of binding of preimmune (—■—) and immune (—□—) IgG by 100 µM peroxynitrite-pmodified H2A. The microtitre wells were coated with 100 µM peroxynitrite–modified H2A.

## Use of anti-100 μM peroxynitrite-modified H2A IgG antibodies to study epitope sharing by other proteins modified by peroxynitrite

That the experimentally produced antibodies against 100 μM peroxynitrite–modified H2A histone are specific has been clearly shown by inhibition studies (Fig. 22**b** and Fig. 24). Native H2A histone used as competitor of anti-100 μM peroxynitrite-modified H2A IgG showed 52% inhibition in antibody binding (Fig. 25**a**). It indicates that not all epitopes typical of native H2A histone have been polymerized into neo-epitopes upon peroxynitrite modification. It means peroxynitrite–modified H2A histone still has some old epitopes which are scattered among neo-epitopes. Therefore, immunization with peroxynitrite–modified histone may produce polyspecific antibodies which can recognize both old and neo-epitopes or altogether there are two types of antibodies, one recognizing nitrated neo-epitopes and other binding exclusively with old epitopes. Binding of anti-100 μM peroxynitrite-modified H2A IgG antibodies was also carried out with an array of peroxynitrite-modified proteins including H1, H2B and H3 histones (Fig. 25**b**,**c**,**d**–28). Results of cross-reaction studies have been summarized in Table 12. This study suggests that nitrated epitopes of different proteins (or amino acids) share common properties. Furthermore, among peroxynitrite-modified amino acids, nitrated-tyrosine showed 70% inhibition in antibody binding followed by nitrated-phenylalanine and nitrated-tryptophan. Participation of nitrated-tyrosine (nitrotyrosine) as preferred substrate for anti-100 μM peroxynitrite–modified H2A IgG antibodies points out the significance of nitrotyrosine in immunogenicity.

## Gel retardation assay

Antibody specificity for the immunogen was evaluated by mobility shift of antigen-antibody complex in denaturing polyacrylamide gel. Constant amount of native H2A histone or peroxynitrite-modified H2A was incubated with varying amounts of anti-100 μM peroxynitrite-modified H2A IgG antibodies for 2 hr at 37°C and overnight at 4°C. The antigen-antibody complex thus formed was then electrophoresed on 10% SDS-polyacrylamide for 4 hr at 80 V and stained with silver nitrate. Formation of high molecular mass immune complex with retarded mobility was observed (Fig. 29**b**). Increasing antibody amount has produced

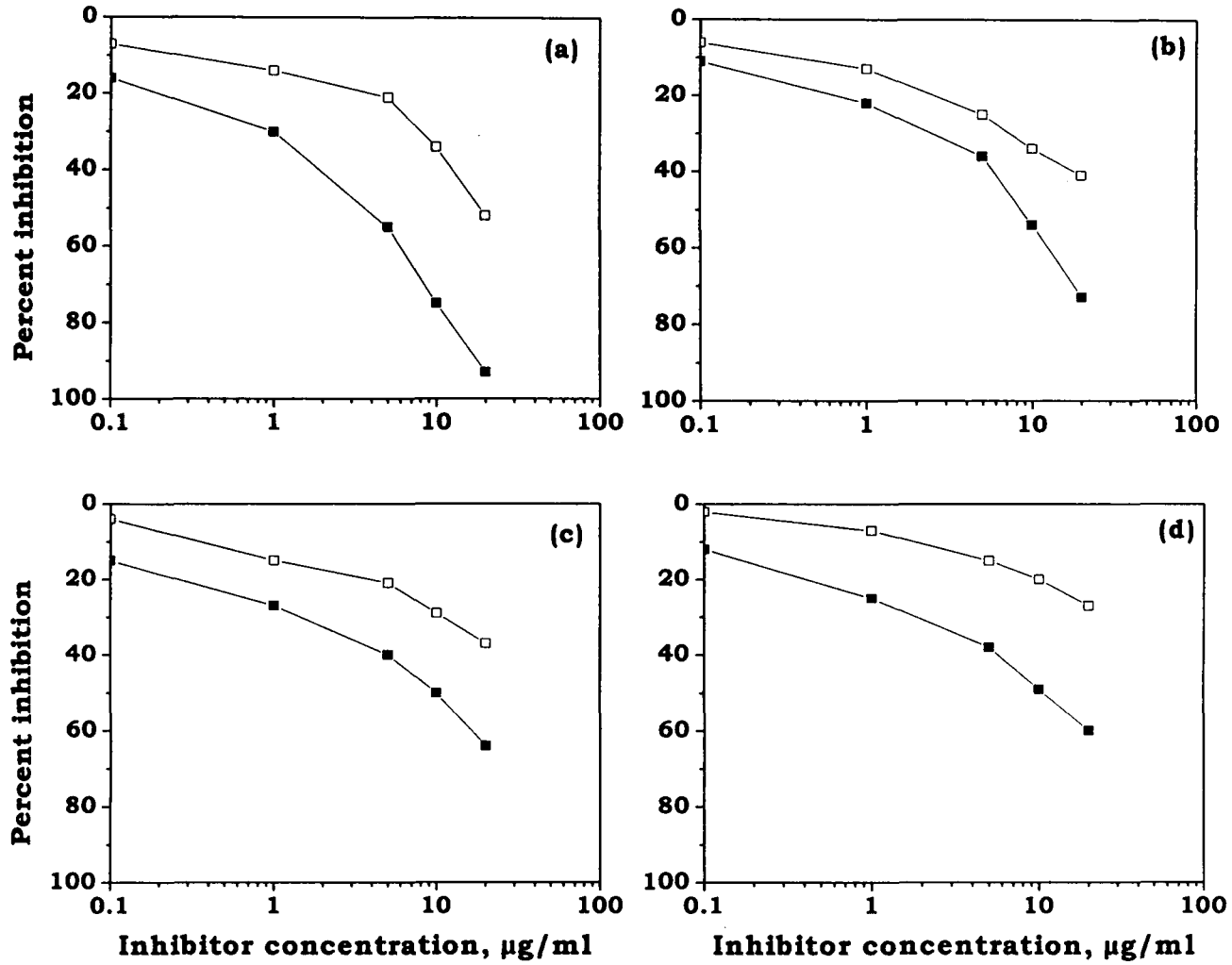



**Fig. 25** Inhibition ELISA of anti-peroxynitrite–modified H2A IgG antibodies binding by different inhibitors. The microtitre wells were coated with peroxynitrite–modified H2A histone.

**(a)** Native H2A (—□—) and peroxynitrite–modified H2A (—■—).

**(b)** Native H2B (—□—) and peroxynitrite–modified H2B (—■—).

**(c)** Native H3 (—□—) and peroxynitrite–modified H3 (—■—).

**(d)** Native H1 (—□—) and peroxynitrite–modified H1 (—■—).

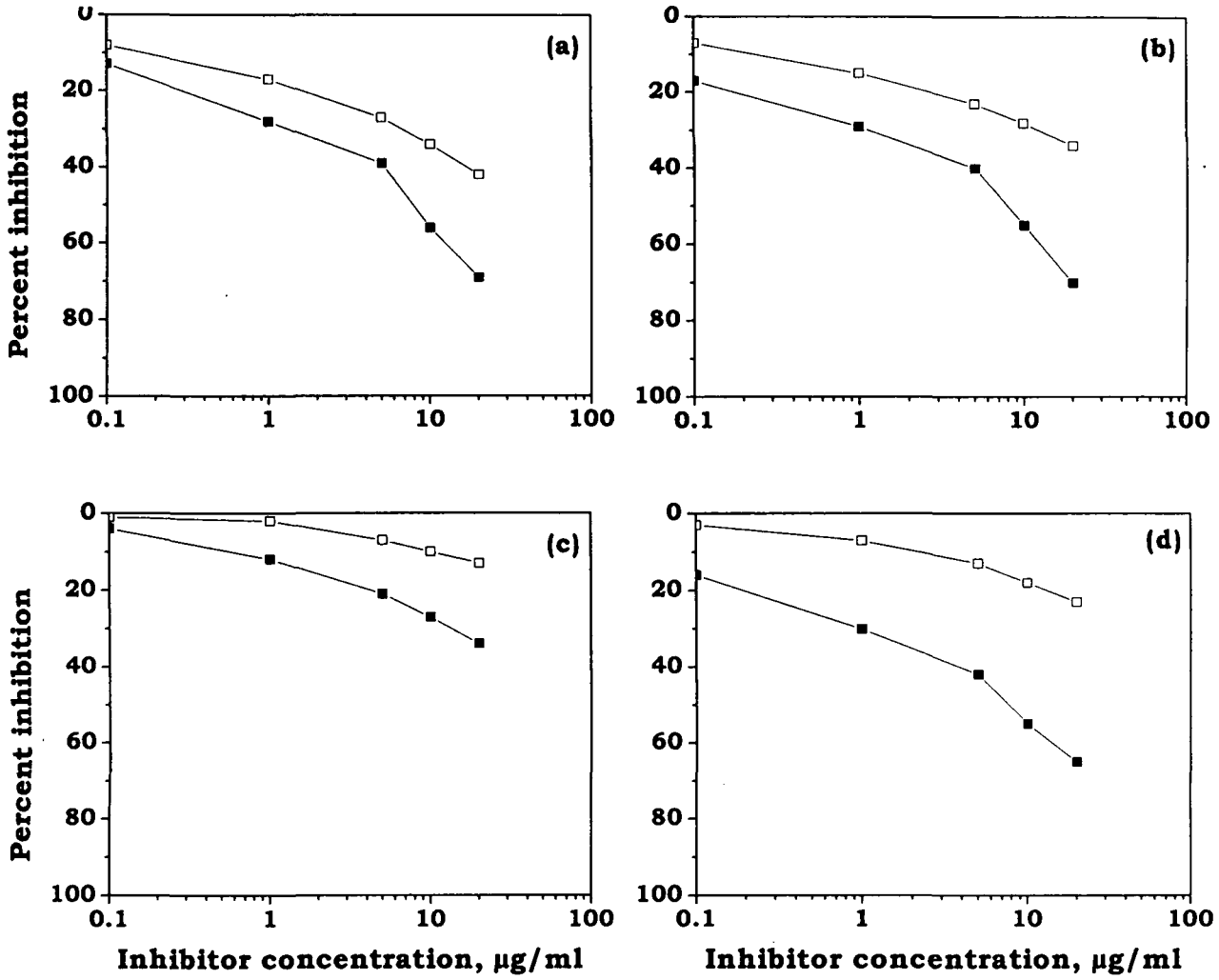



**Fig. 26** Inhibition ELISA of anti-peroxynitrite-modified H2A IgG antibodies binding by different inhibitors. The microtitre wells were coated with peroxynitrite-modified H2A histone.

**(a)** Native human IgG (—□—) and peroxynitrite-modified human IgG (—■—).

**(b)** Native tyrosine (—□—) and peroxynitrite-modified tyrosine (—■—).

**(c)** Native SOD (—□—) and peroxynitrite-modified SOD (—■—).

**(d)** Native HSA (—□—) and peroxynitrite-modified HSA (—■—).

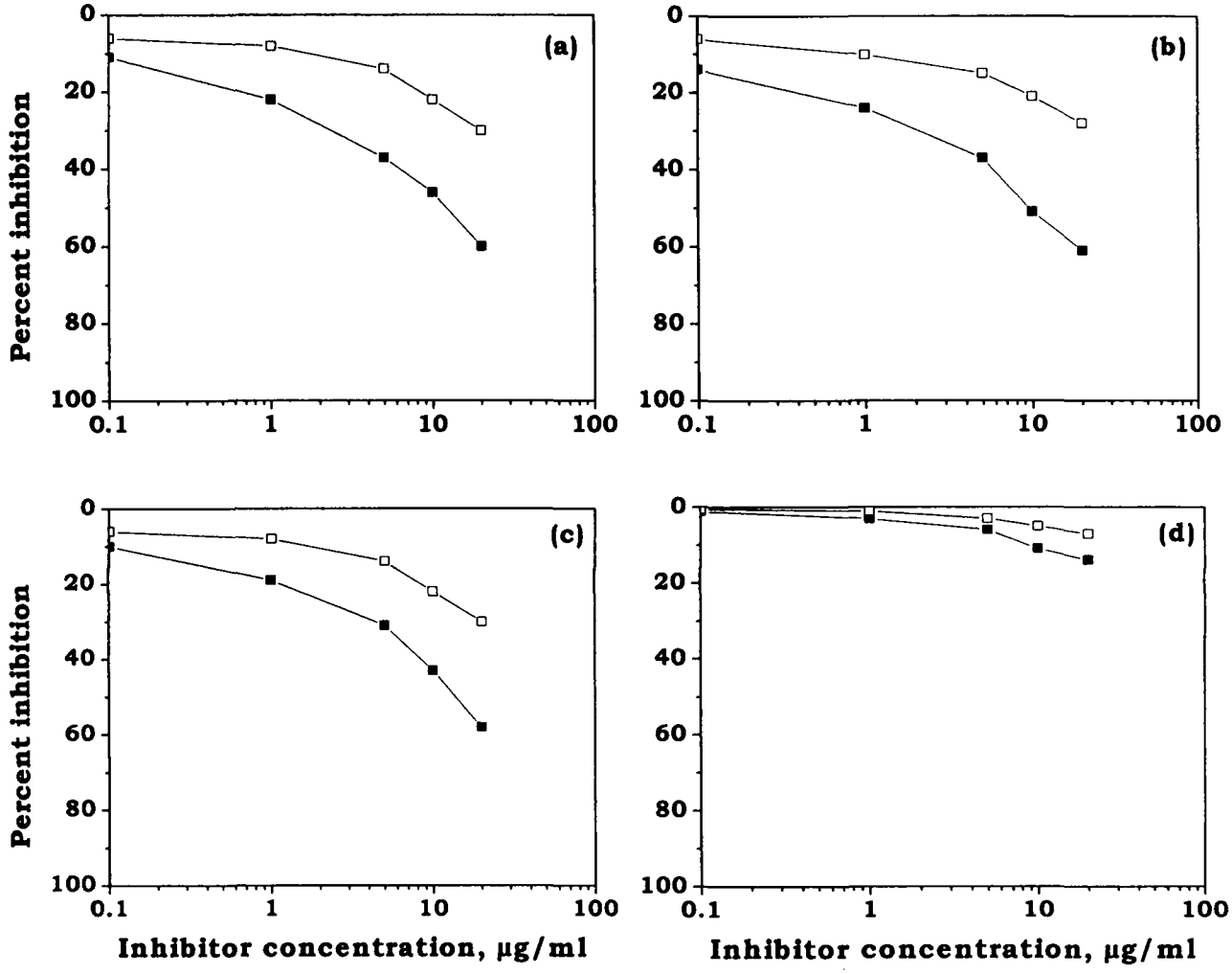



**Fig. 27** Inhibition ELISA of anti-peroxynitrite–modified H2A IgG antibodies binding by different inhibitors. The microtitre wells were coated with peroxynitrite–modified H2A histone.

**(a)** Native catalase (—□—) and peroxynitrite–modified catalase (—■—).

**(b)** Native LDL (—□—) and peroxynitrite–modified LDL (—■—).

**(c)** Native trypsin (—□—) and peroxynitrite–modified trypsin (—■—).

**(d)** Native tryptophan (—□—) and peroxynitrite–modified tryptophan (—■—).

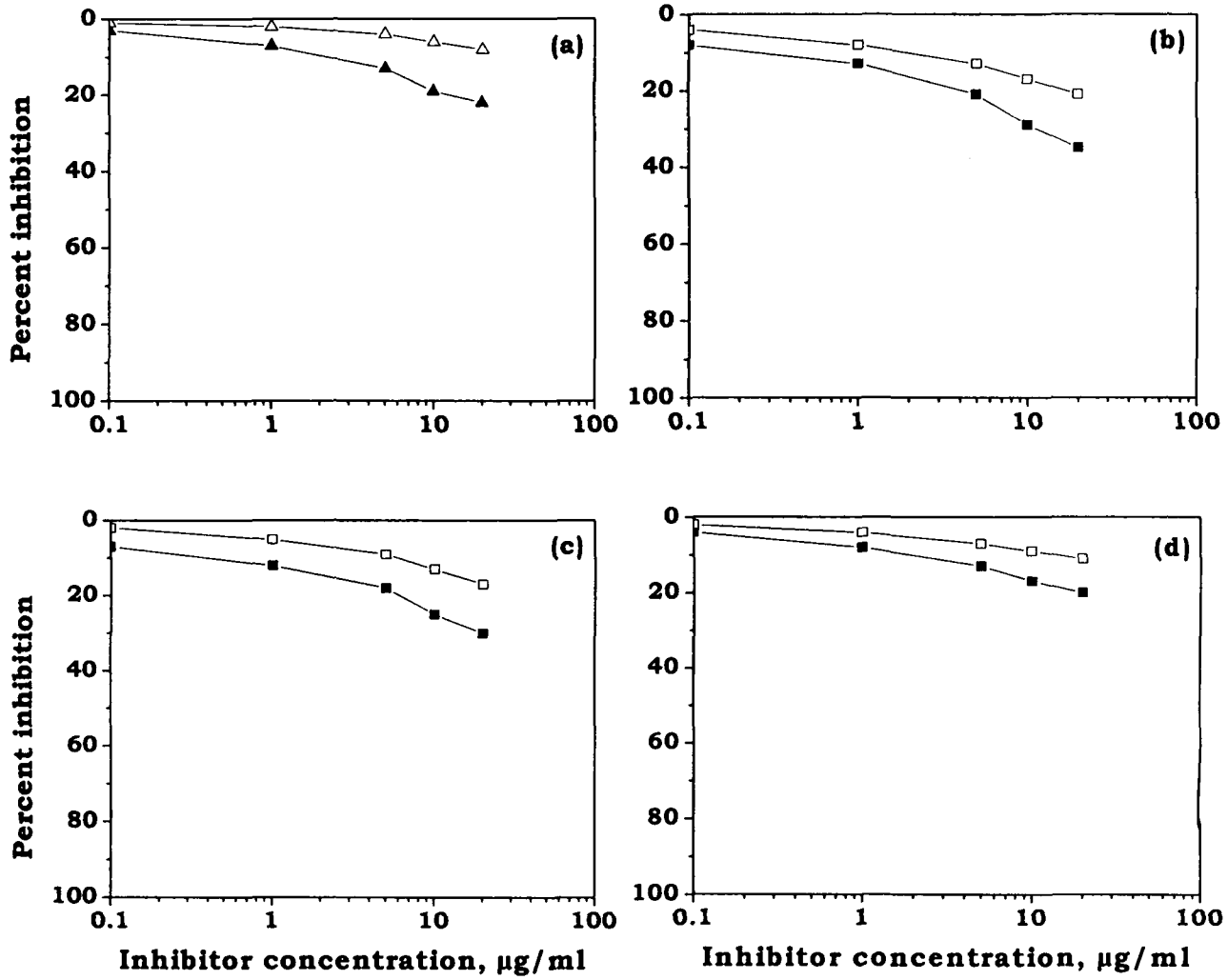



**Fig. 28** Inhibition ELISA of anti-peroxynitrite–modified H2A IgG antibodies binding by different inhibitors. The microtitre wells were coated with peroxynitrite–modified H2A histone.

**(a)** Native phenylalanine (—□—) and peroxynitrite–modified phenylalanine (—■—).

**(b)** Native pDNA (—□—) and peroxynitrite–modified pDNA (—■—).

**(c)** Native calf thymus DNA (—□—) and peroxynitrite–modified calf thymus DNA (—■—).

**(d)** Native yeast RNA (—□—) and peroxynitrite–modified yeast RNA (—■—).

## Table 12

**Cross reactions of peroxynitrite–modified H2A IgG antibodies[#]**

| Inhibitor | Maximum percent inhibition at 20 µg/ml |
|---|---|
| Peroxynitrite–modified H2A | 93 |
| Native H2A | 52 |
| Peroxynitrite–modified H2B | 73 |
| Native H2B | 41 |
| Peroxynitrite–modified H3 | 64 |
| Native H3 | 37 |
| Peroxynitrite–modified H1 | 60 |
| Native H1 | 27 |
| Peroxynitrite–modified IgG | 69 |
| Native human IgG | 27 |
| 3–nitrotyrosine | 70 |
| Tyrosine | 34 |
| Peroxynitrite–modified SOD | 34 |
| Native SOD | 10 |
| Peroxynitrite–modified HSA | 65 |
| Native HSA | 23 |
| Peroxynitrite–modified Catalase | 60 |
| Native Catalase | 30 |
| Peroxynitrite–modified LDL | 61 |
| Native LDL | 28 |
| Peroxynitrite–modified Trypsin | 58 |
| Native trypsin | 20 |
| Peroxynitrite–modified Tryptophan | 14 |
| Native tryptophan | 07 |
| Peroxynitrite–modified Phenylalanine | 22 |
| Native phenylalanine | 08 |
| Peroxynitrite–modified human pDNA | 35 |
| Native human pDNA | 21 |
| Peroxynitrite–modified calf thymus DNA | 30 |
| Native calf thymus DNA | 17 |
| Peroxynitrite–modified yeast RNA | 20 |
| Native yeast RNA | 11 |

[#] Wells were coated with 100 µM peroxynitrite–modified H2A histone.

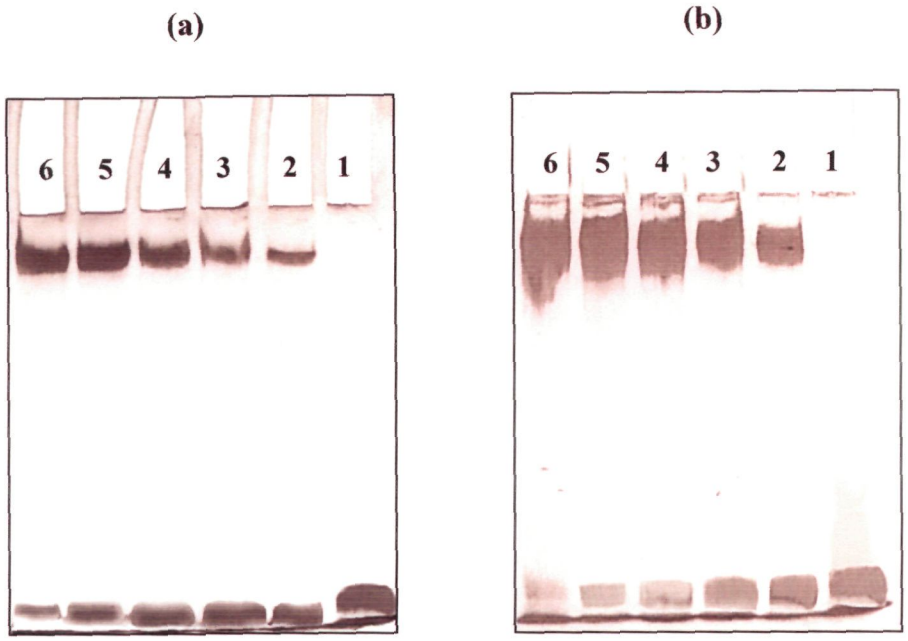



**Fig. 29** Mobility shift assay of anti-100 μM peroxynitrite–modified-H2A IgG antibodies in SDS–polyacrylamide gel. Electrophoresis was performed on 10% gel for 4 hr at 80 Volts.

**(a)** Native H2A (25 μg, lane 1) was incubated with 10, 20, 30, 40 and 50 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated for 2 hr at 37°C and overnight at 4°C.

**(b)** Peroxynitrite–modified H2A (25 μg, lane 1) was incubated with 10, 20, 30, 40 and 50 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated under identical conditions.

corresponding increase in immune complex as evident from intensity of band. The free antigen showed proportional decrease of intensity. The mobility shift results indicated that induced antibodies against 100 μM peroxynitrite-modified H2A are specific but also recognizing the epitopes on native H2A (Fig. 29**a**).

## Studies on systemic lupus erythematosus patients

*Direct binding ELISA of SLE sera with native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A*

All 50 SLE samples included in this study were pre-tested for anti-DNA autoantibodies and found to be positive. Age- and sex- matched sera from apparently healthy individuals served as control. The sera were diluted 1:100 and subjected to direct binding ELISA on microtitre wells separately coated with equal amounts of native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A histone (Fig. 30–32). Twenty SLE sera (40%), *labeled, showed higher binding with peroxynitrite–modified H2A histone compared to native DNA or native H2A histone. No appreciable binding was observed with the pooled sera of normal subjects. These observations are important in view of the immunogenic nature of peroxynitrite-modified H2A histone and reported incidence of hypernitrotyrosinemia and anti-histone antibodies in some SLE patients.

*Enzyme immunoassay of SLE IgG with native DNA, native H2A histone and 100 μM peroxynitrite-modified H2A*

IgG were purified from twenty SLE sera which showed higher binding with peroxynitrite-modified H2A histone in direct binding ELISA. All SLE IgG were first subjected to direct binding assay on microtitre wells coated with 100 μM peroxynitrite-modified H2A. This helped us to work out the amount of IgG antibodies required for antigen saturation. The direct binding profile of one SLE IgG has been shown in Fig. 33.

The fine antigenic specificity of isolated SLE IgG was evaluated using native DNA, native H2A and 100 μM peroxynitrite-modified H2A as inhibitors. Purified SLE IgG was separately mixed with DNA, H2A and 100 μM peroxynitrite–modified H2A (0-20 μg/ml) and incubated for 2 hr at 37°C and overnight at 4°C. The resulting complex was coated instead of only serum/IgG.

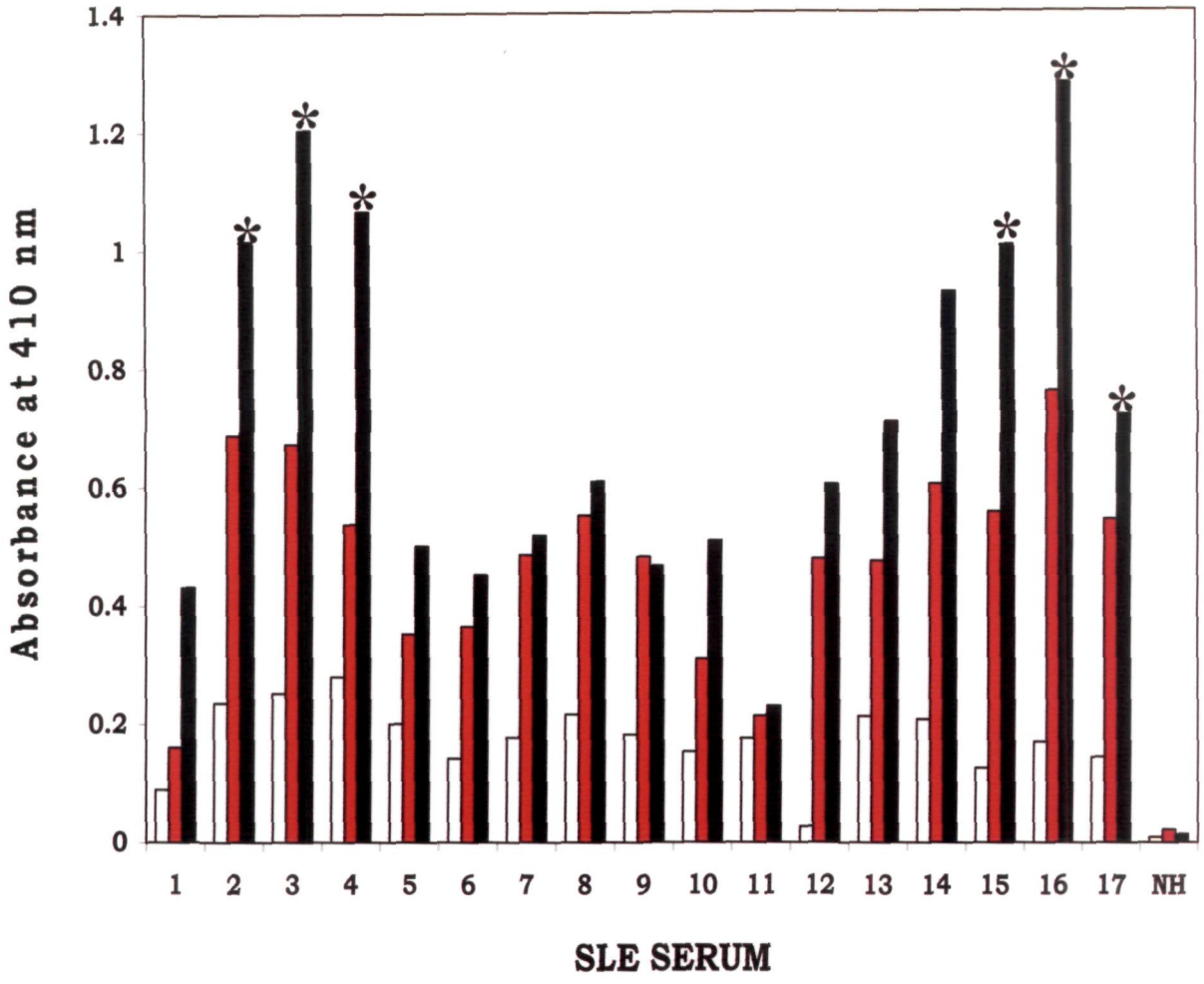



**Fig. 30** Binding of 1:100 diluted SLE sera to native DNA (—■—), native histone H2A (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

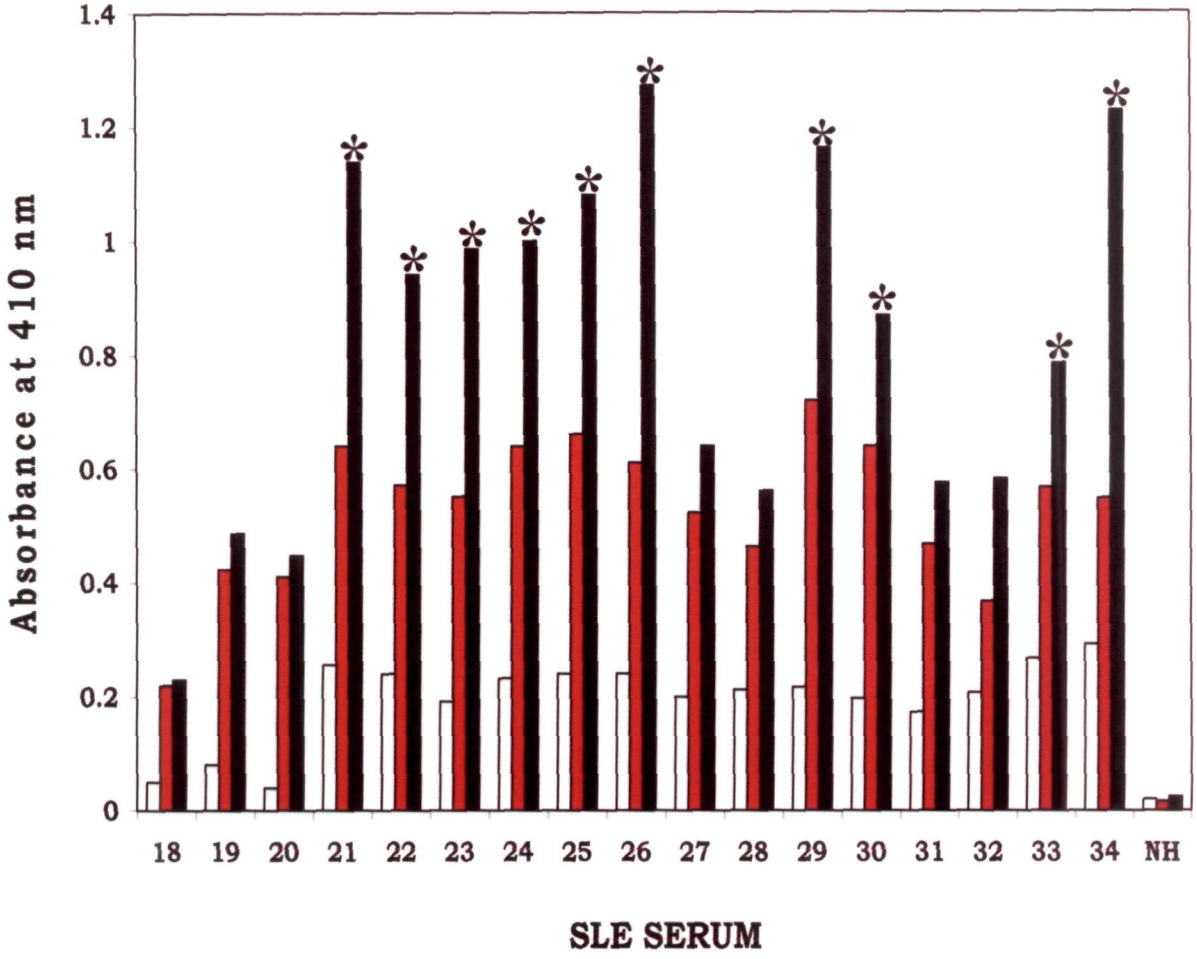



**Fig. 31** Binding of 1:100 diluted SLE sera to native DNA (—■—), native histone H2A (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

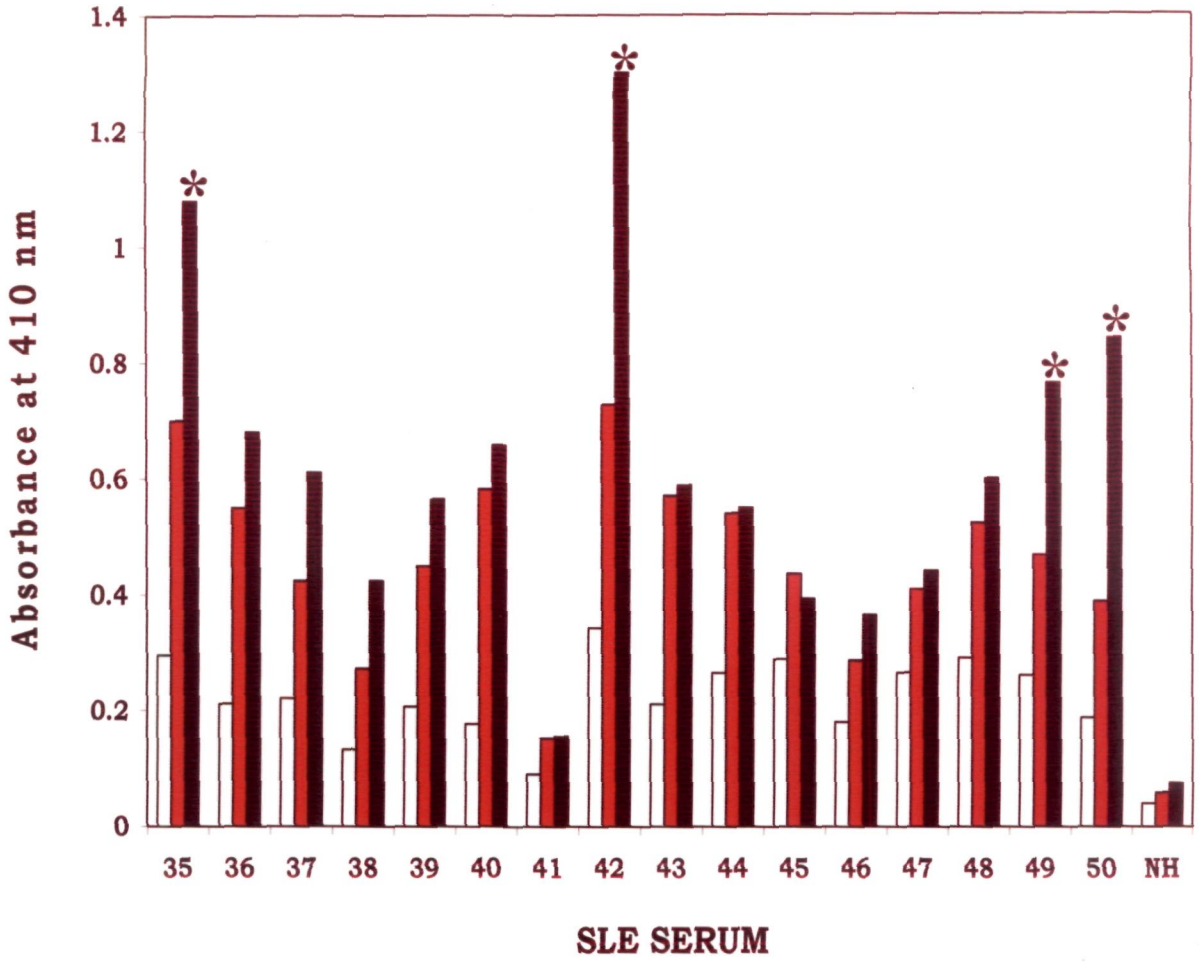



**Fig. 32** Binding of 1:100 diluted SLE sera to native DNA (—■—), native histone H2A (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

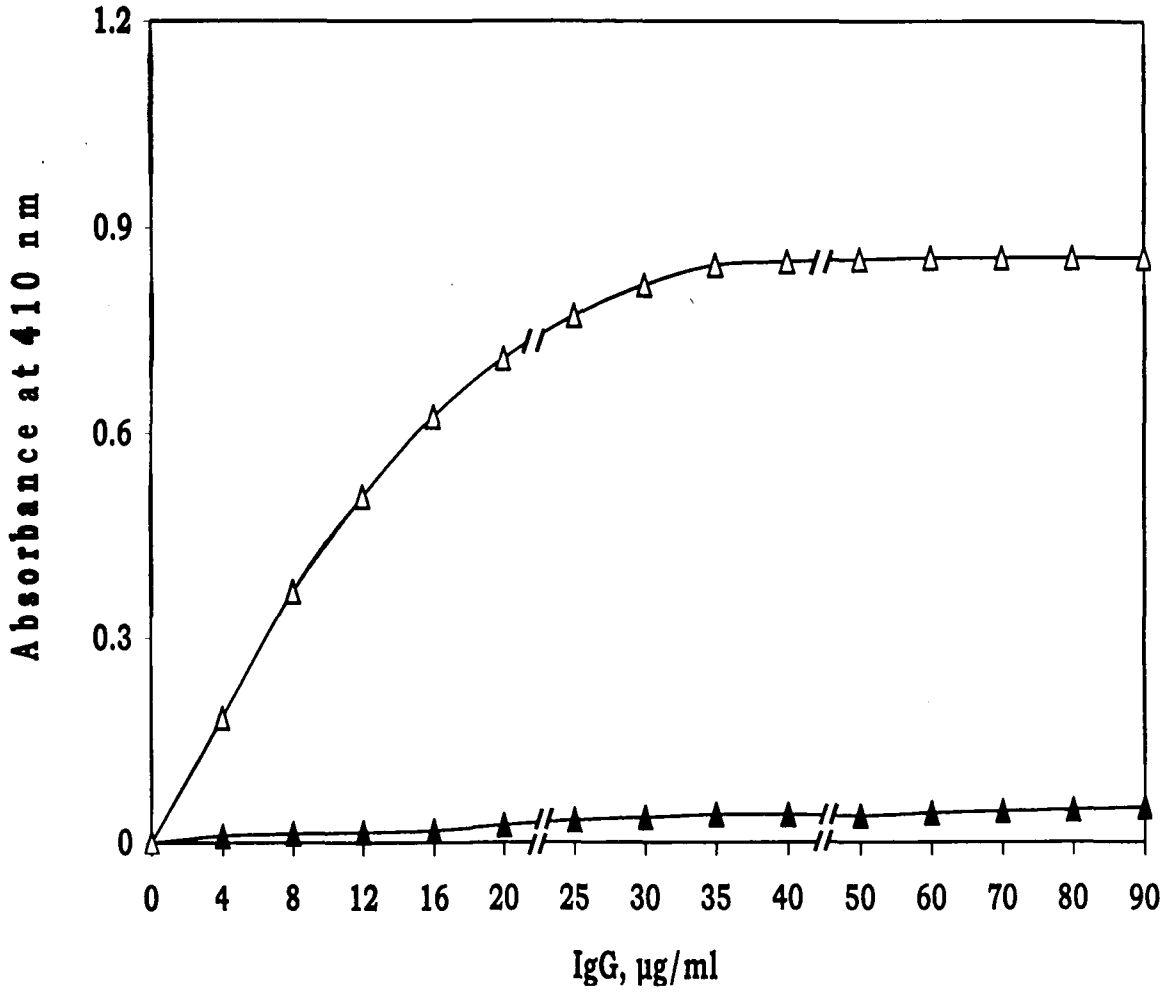



**Fig. 33** Direct binding ELISA of a purified SLE IgG (—Δ—) and normal human IgG (—▲—) on microtitre wells coated with 100 µM peroxynitrite–modified H2A histone.

The remaining steps were same as described for direct binding ELISA. The interaction of inhibitors with different SLE IgG has been represented as percent inhibition in antibody binding with coated antigen (Fig. 34–38). The peroxynitrite–modified H2A histone emerged as the most powerful inhibitor followed by native DNA and native H2A. The summary of inhibition studies has been complied in Table 13. Inter-comparison of data by a statistical method yielded a *p*-value of <0.001 for peroxynitrite-modified H2A vs native DNA or native histone. This points out that the data are statistically significant. The immunogenic nature of peroxynitrite-modified H2A (or other protein) and the reported incidence of anti-histone antibodies and hypernitrotyrosinemia in SLE patients advocates possibile role of peroxynitrite–modified protein antigens in SLE initiation in a sub-population of patients.

*Determination of protein carbonyl content in SLE sera*

Carbonyl contents in 12 SLE sera (no. 2–4, 14–16, 21, 25, 26, 29, 34 and 42) and 12 normal human sera were determined to assess the effect of *in vivo* oxidative stress in normal subjects and SLE patients. The average carbonyl contents of 12 SLE sera was found to be 2.85 ± 0.21 nmole/mg protein compared to 1.95 ± 0.20 nmole/mg protein in healthy subjects (Fig. 39). The statistical analysis of data revealed that SLE samples had significantly higher content of carbonyls ($p < 0.05$) compared to normal sera.

*Estimation of nitrotyrosine*

First of all, a proper control was prepared by mixing equal amount of ten serum samples of apparently normal human subjects. Fig. 40 represents the HPLC chromatogram of pooled control serum. We did not observe nitrotyrosine peak (corresponding to ~12.25 min retention time) in the control serum. It may be recalled that a standard solution of 3-nitrotyrosine processed under identical experimental conditions gave a clear cut peak having a retention time of ~12.25 min (Fig. 7). Results of HPLC analysis of 10 SLE samples are given in Fig. 41–45. All samples were positive for nitrotyrosine because we observed peaks matching with the retention time of 3-nitrotyrosine. The concentration of 3-nitrotyrosine in SLE samples were calculated from the calibration curve of standard 3-nitrotyrosine and the data are compiled in Table 14.

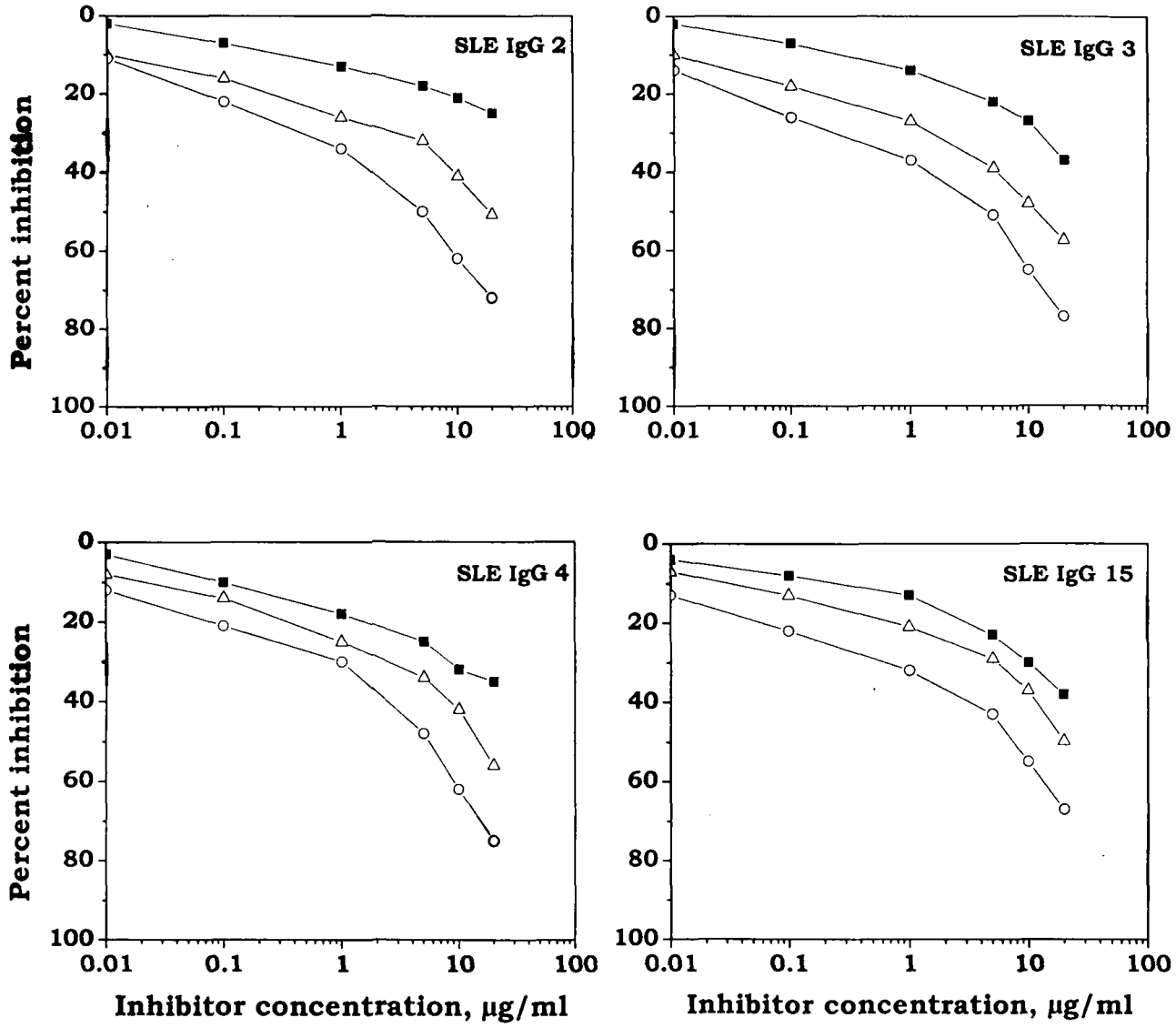



**Fig. 34** Inhibition of SLE IgG (isolated from sera **2**, **3**, **4** and **15**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite-modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

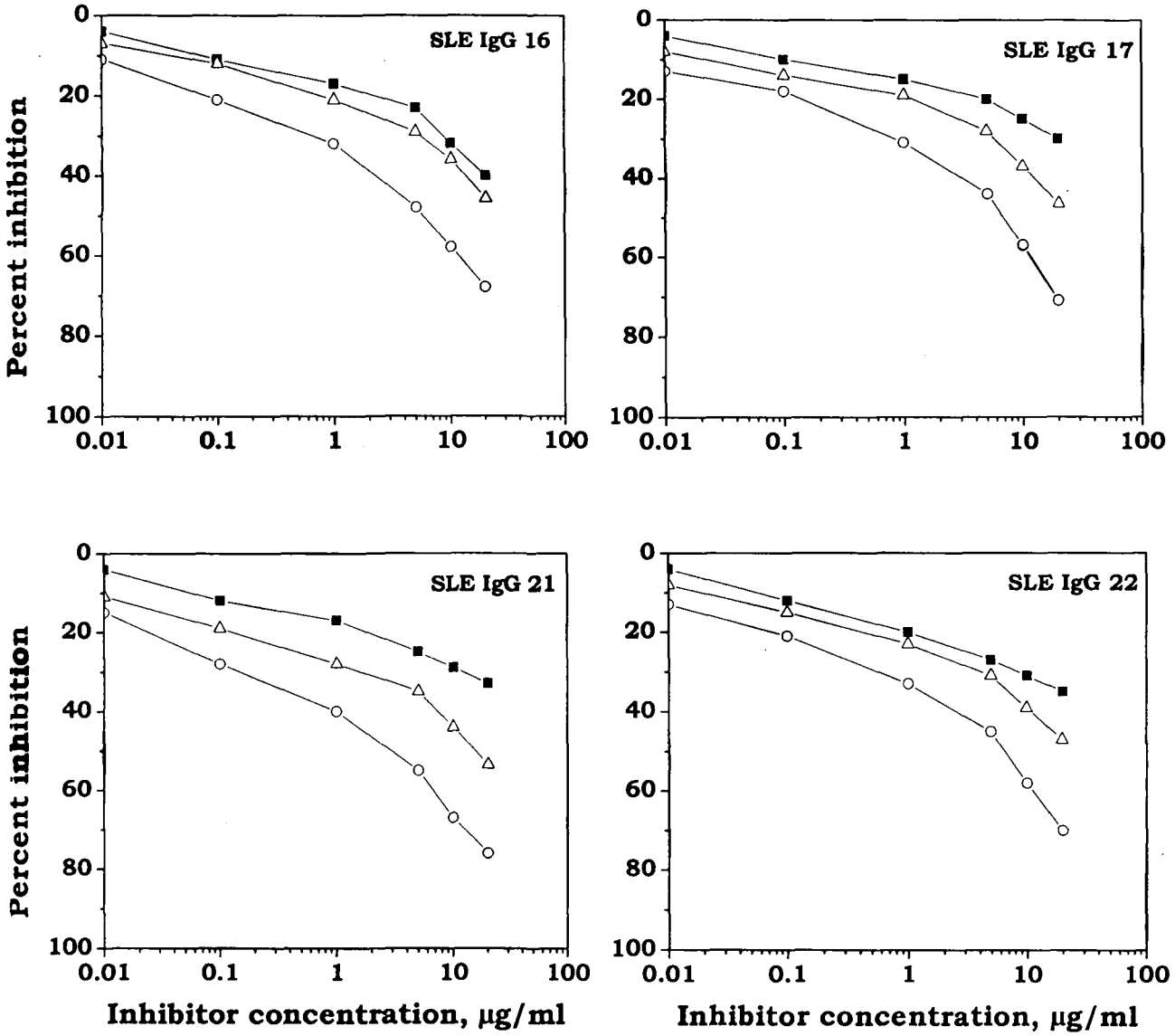



**Fig. 35** Inhibition of SLE IgG (isolated from sera **16, 17, 21** and **22**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 μM peroxynitrite-modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 μg/ml).

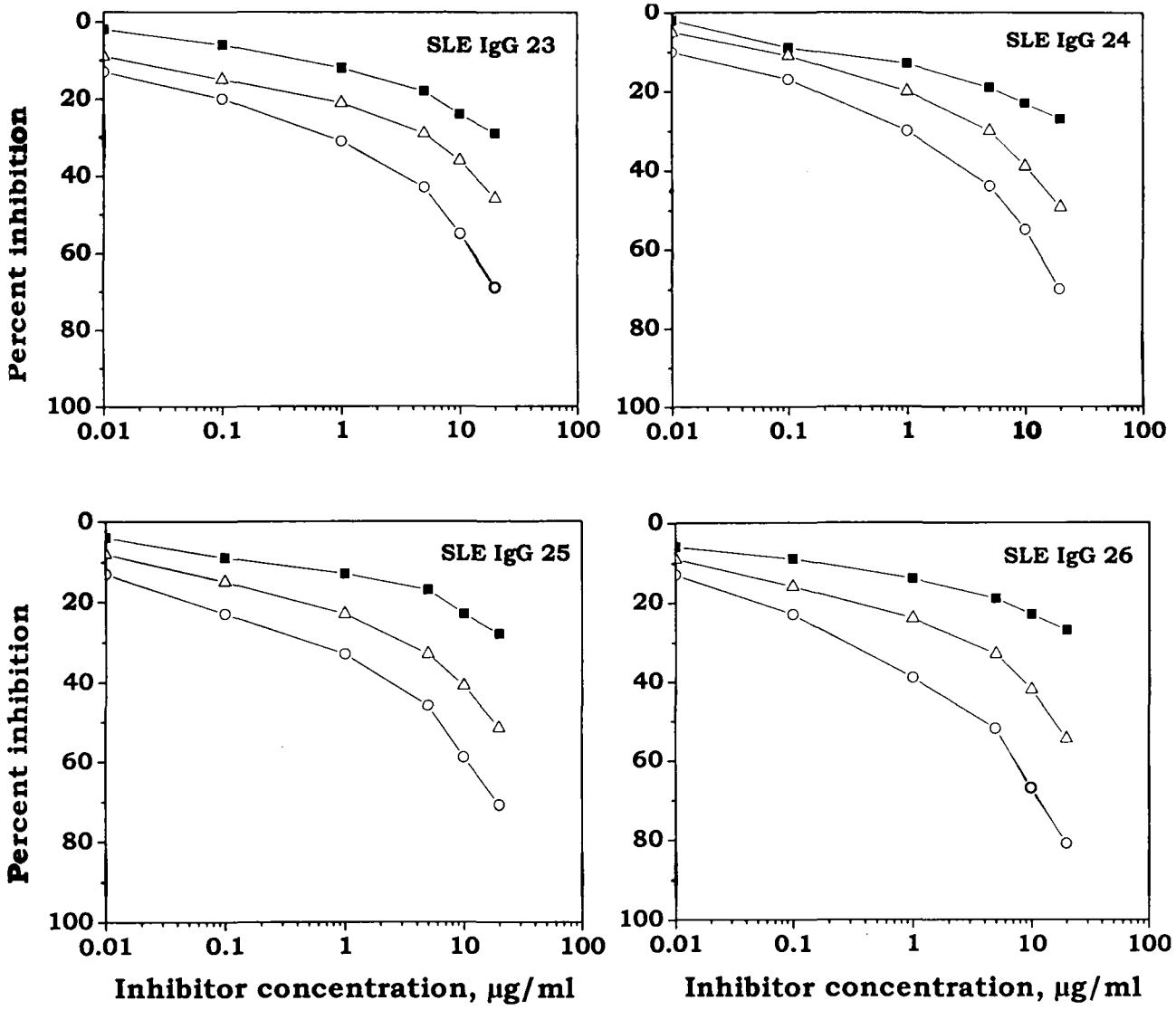



Fig. 36 Inhibition of SLE IgG (isolated from sera **23, 24, 25** and **26**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite-modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

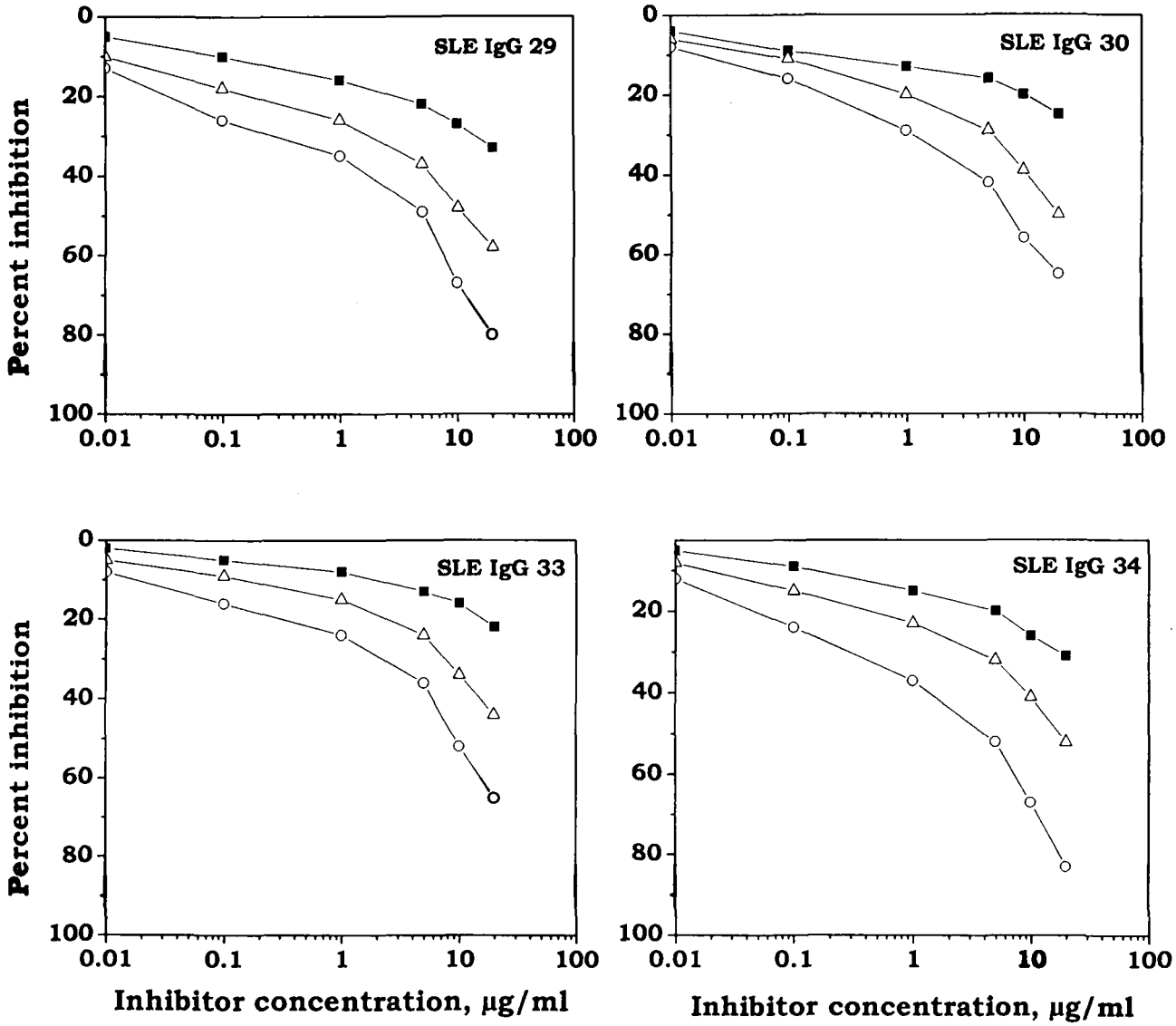



Fig. 37 Inhibition of SLE IgG (isolated from sera **29, 30, 33** and **34**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite–modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

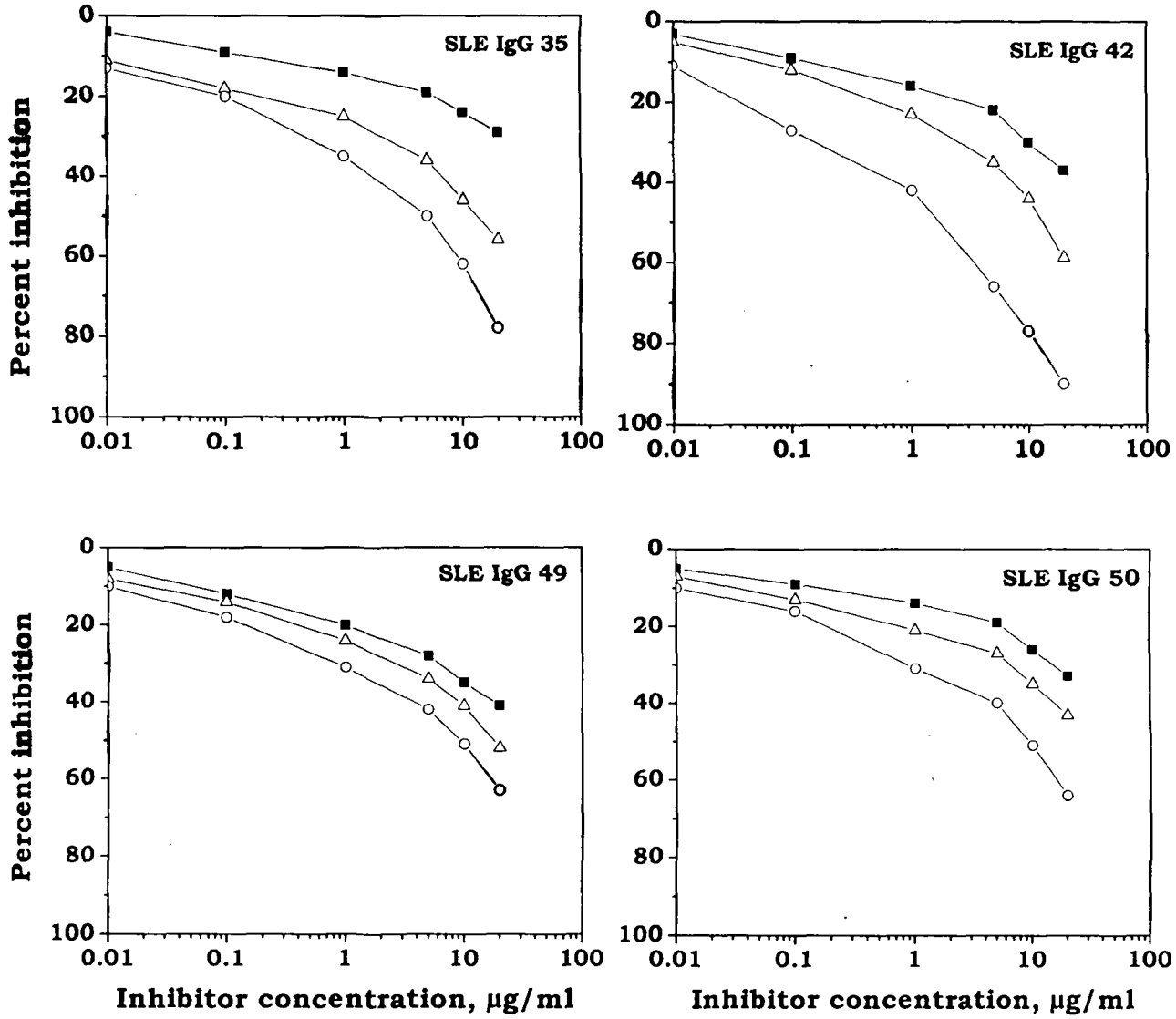



**Fig. 38** Inhibition of SLE IgG (isolated from sera **35, 42, 49** and **50**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite-modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

## Table 13

**#Inhibition of SLE IgG binding by native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A**

| IgG | Maximum percent inhibition at 20 μg/ml | | |
|---|---|---|---|
| | nDNA | nH2A | Peroxynitrite–modified H2A |
| 01 (2)* | 50.8 | 25.0 | 72.0 |
| 02 (3) | 57.4 | 37.0 | 77.0 |
| 03 (4) | 56.0 | 35.0 | 75.0 |
| 04 (15) | 49.8 | 38.0 | 67.0 |
| 05 (16) | 45.8 | 40.0 | 68.0 |
| 06 (17) | 46.5 | 30.0 | 71.0 |
| 07 (21) | 53.5 | 33.0 | 76.0 |
| 08 (22) | 47.0 | 35.0 | 70.0 |
| 09 (23) | 45.8 | 29.0 | 69.0 |
| 10 (24) | 49.3 | 37.0 | 70.0 |
| 11 (25) | 51.7 | 28.0 | 71.0 |
| 12 (26) | 54.6 | 27.0 | 81.0 |
| 13 (29) | 58.0 | 33.0 | 80.0 |
| 14 (30) | 50.0 | 25.0 | 65.0 |
| 15 (33) | 44.0 | 22.0 | 65.0 |
| 16 (34) | 52.0 | 31.0 | 83.0 |
| 17 (35) | 56.0 | 29.0 | 78.0 |
| 18 (42) | 58.8 | 37.0 | 90.0 |
| 19 (49) | 52.0 | 41.0 | 63.7 |
| 20 (50) | 43.2 | 33.0 | 64.2 |
| **Mean ± S.D.** | **(51.11 ± 4.75)** | **(32.25 ± 5.32)** | **(72.75 ± 7.13)** |

# Microtitre plates were coated with native DNA.

*Numbers in parentheses show the corresponding SLE serum number in Fig. 30–32 that was used to isolate IgG.

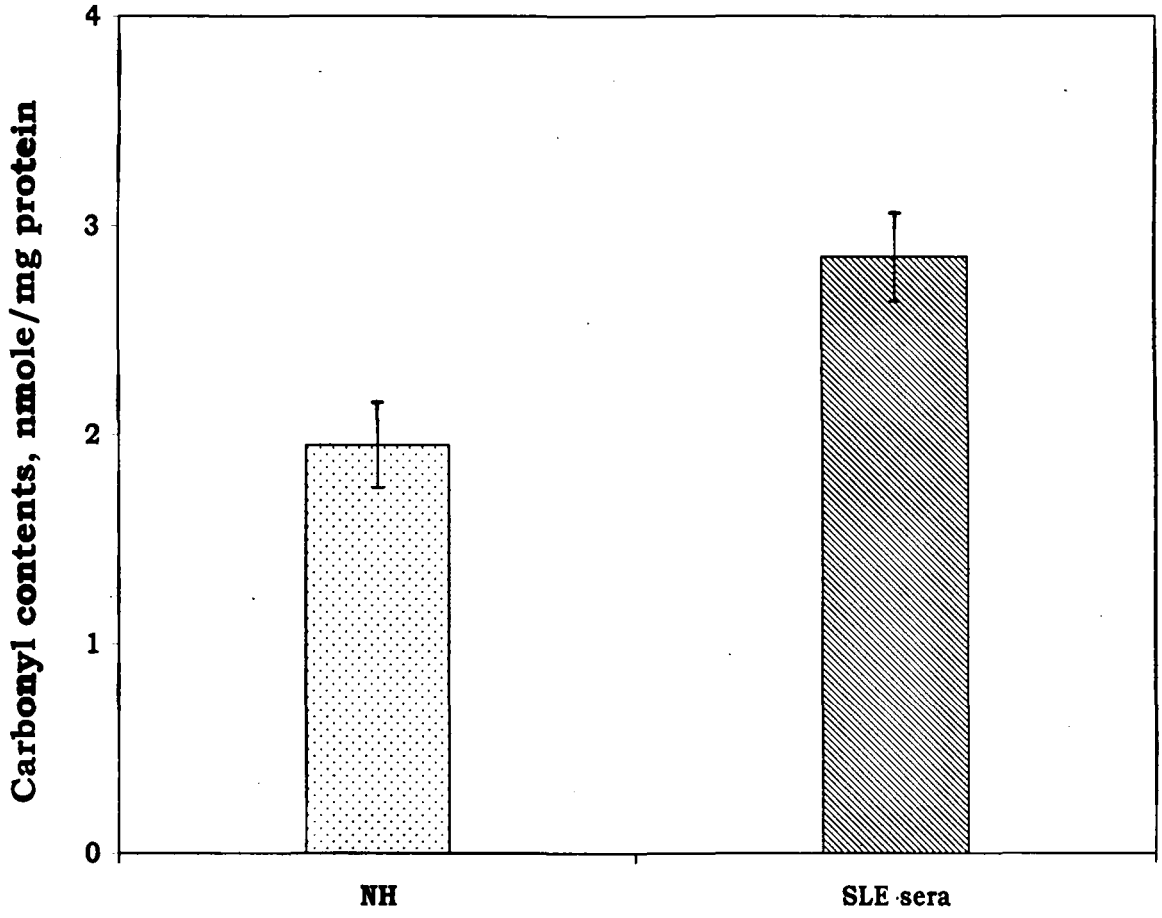



**Fig. 39** Level of carbonyls in SLE and normal human sera. Each histogram represents the mean ± S.D. of 12 samples.

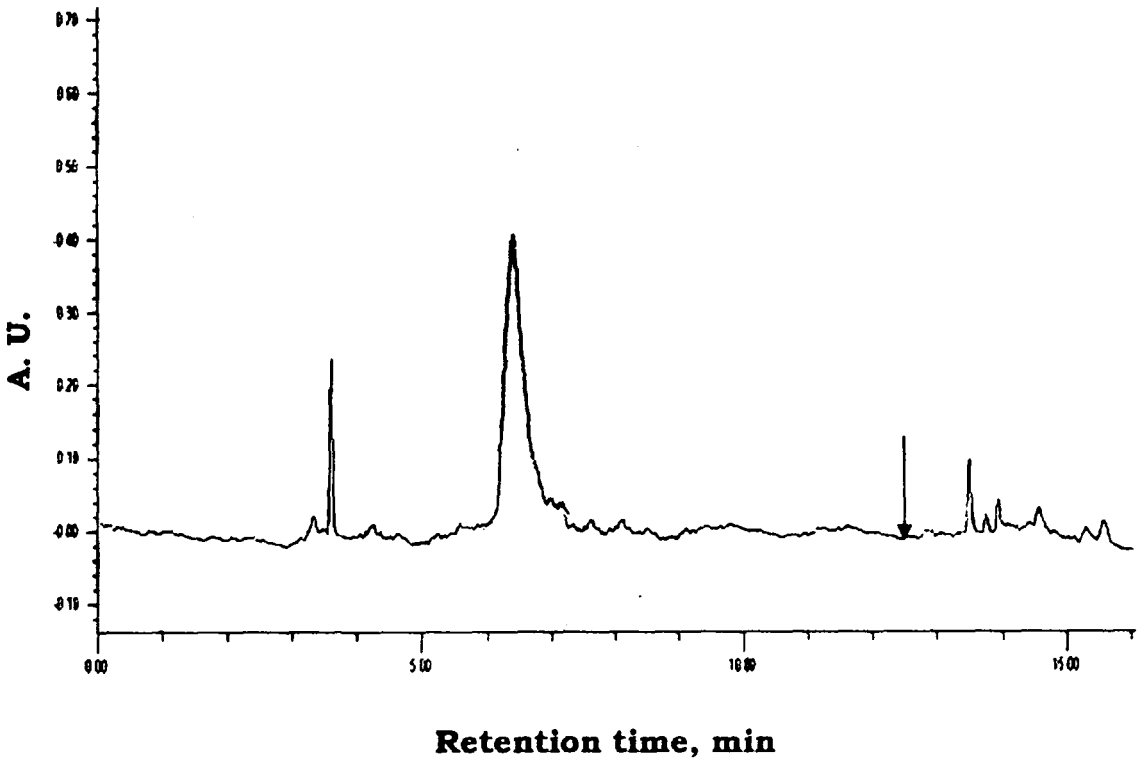



**Fig. 40** HPLC profile of the pooled control serum. The down head arrow points out the absence of nitrotyrosine in the control which otherwise appears at this retention time.

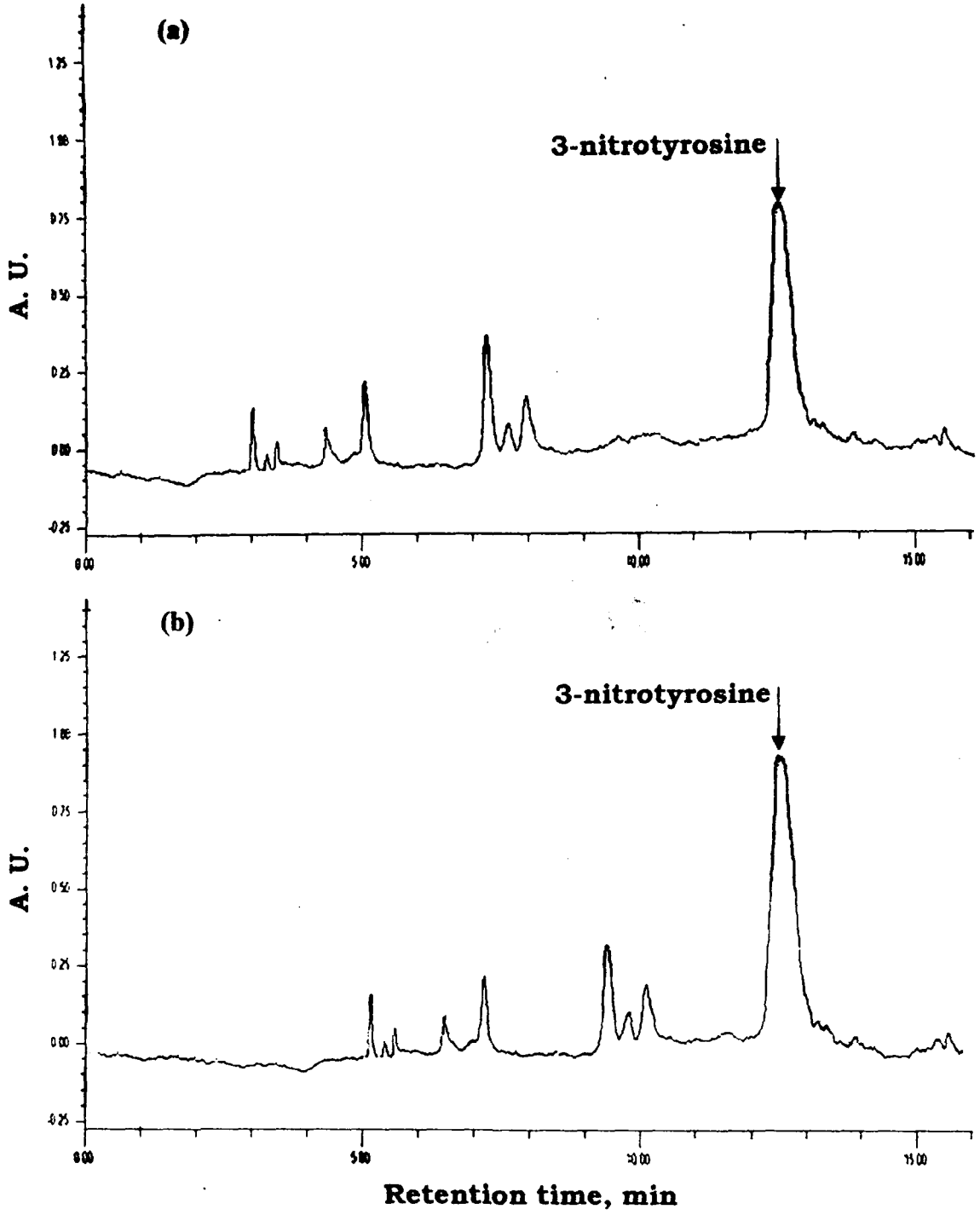



**Fig. 41** HPLC profile of SLE serum sample number 2 **(a)**, and number 3 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

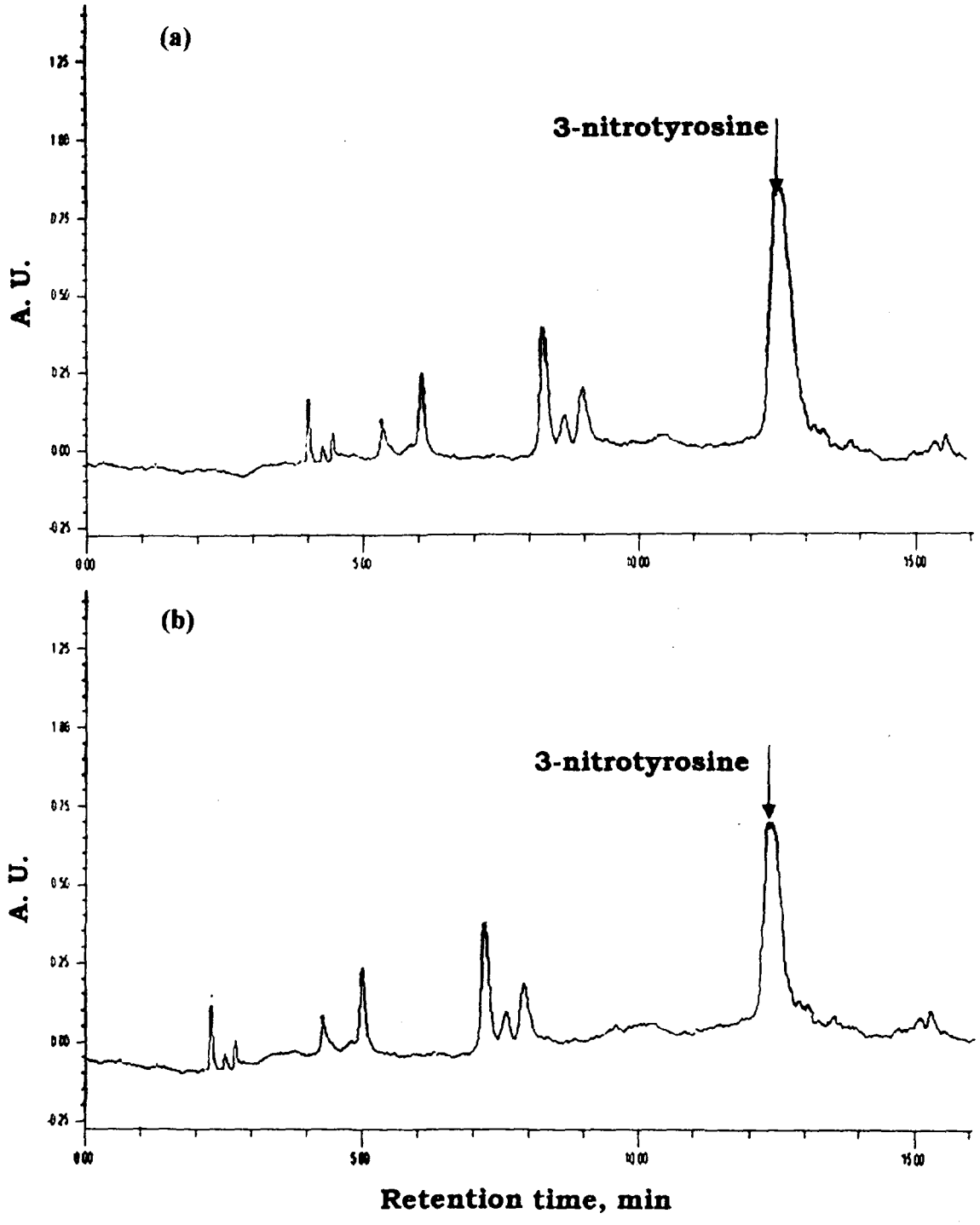



**Fig. 42** HPLC profile of SLE serum sample number 4 **(a)**, and number 16 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

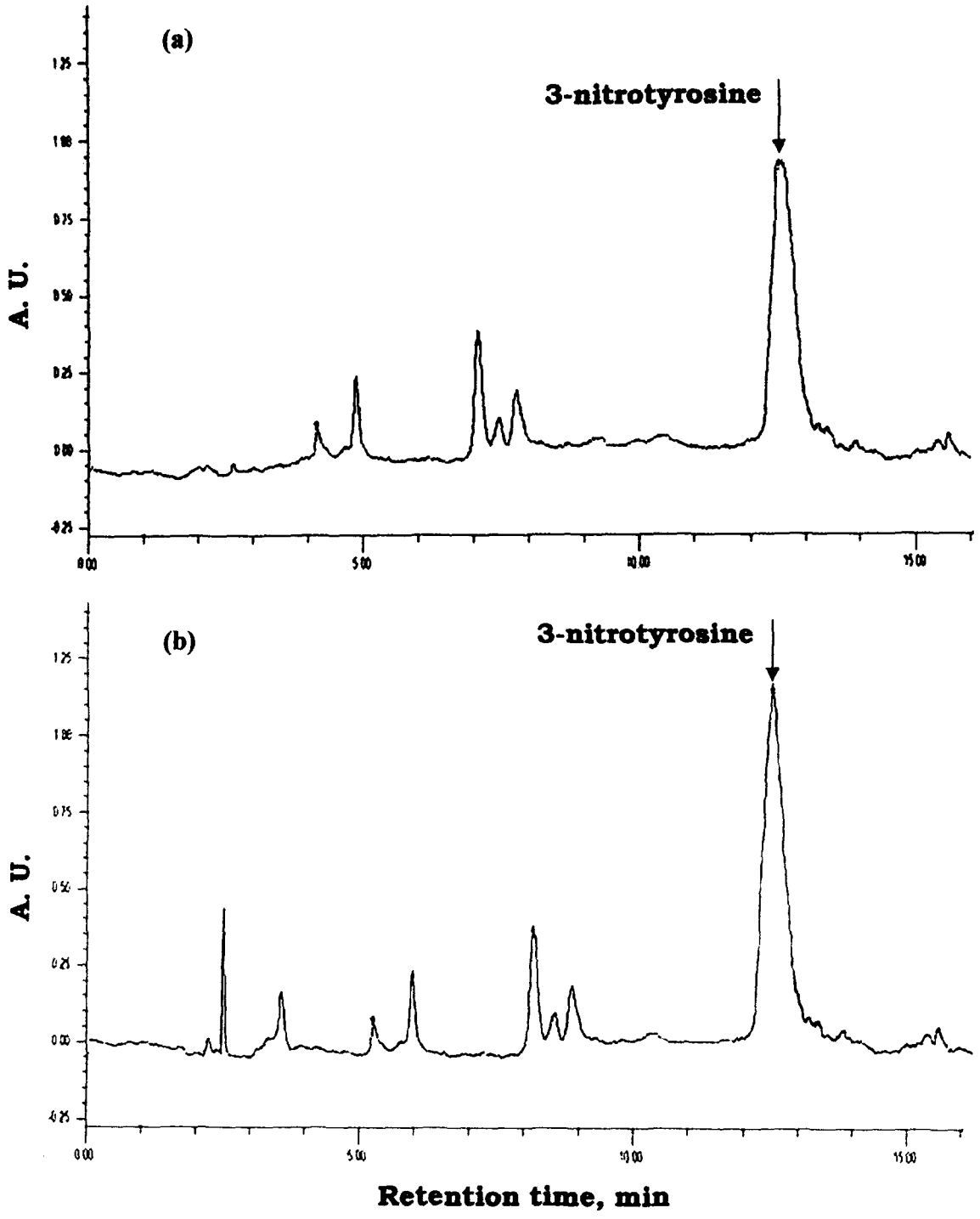



**Fig. 43** HPLC profile of SLE serum sample number 21 **(a)**, and number 26 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

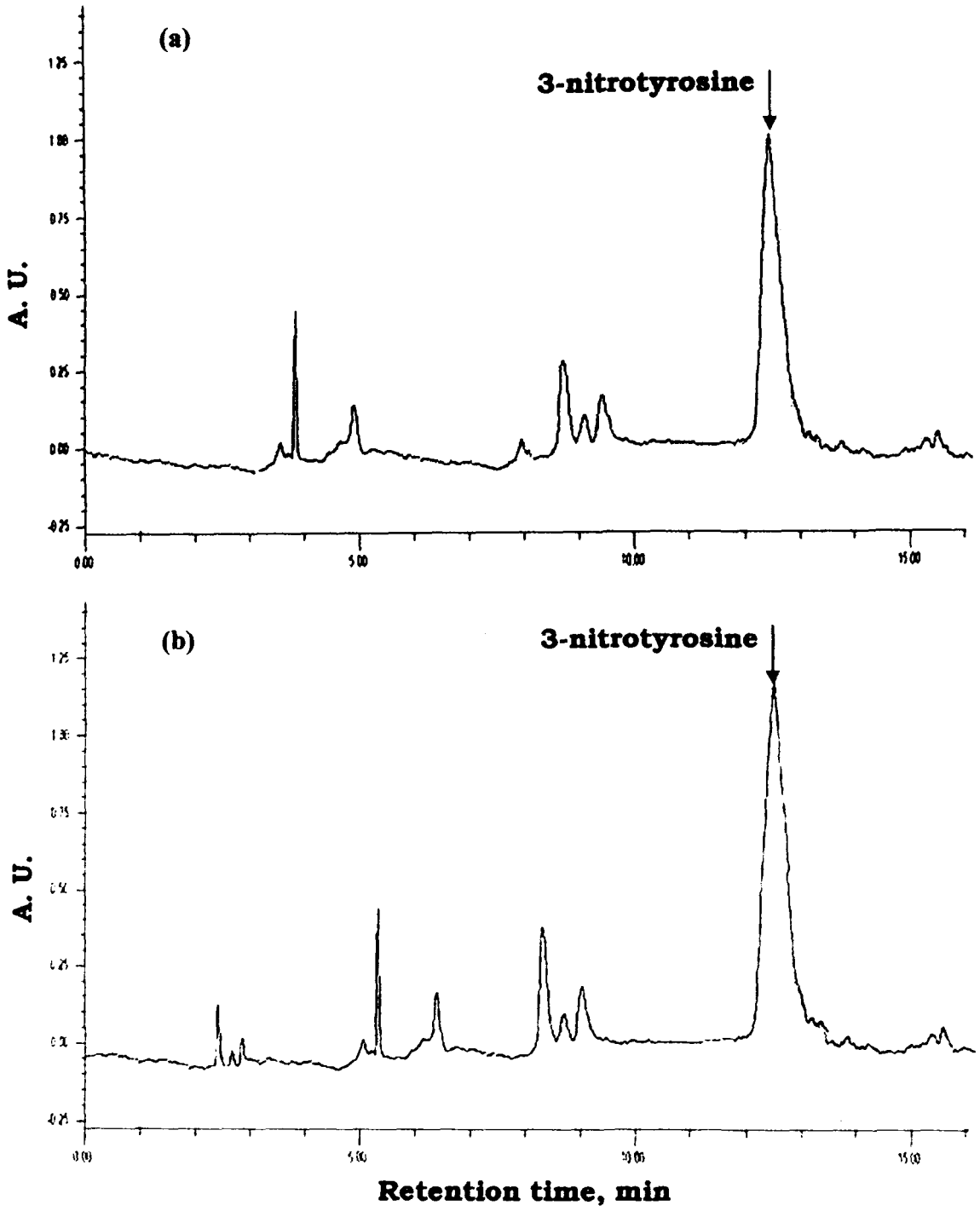



**Fig. 44** HPLC profile of SLE serum sample number 29 **(a)**, and number 34 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

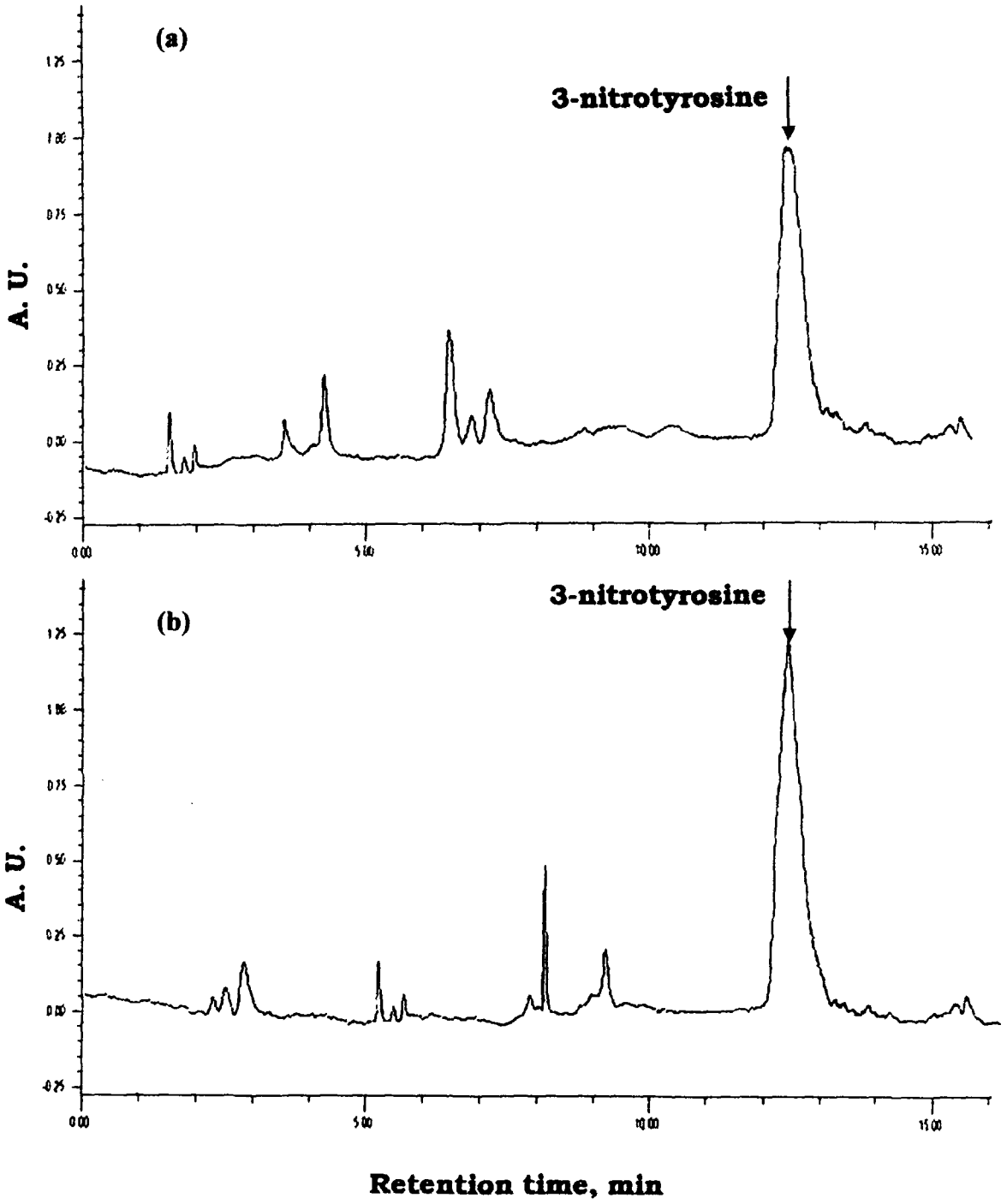



**Fig. 45** HPLC profile of SLE serum sample number 35 **(a)**, and number 42 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

## Table 14

**Quantitative estimation of nitrotyrosine in SLE sera by HPLC**

| SLE serum sample number | Absorbance at 274 nm | µM of niytotyrosine |
|---|---|---|
| 2 | 0.78 | 0.956 |
| 3 | 0.93 | 1.261 |
| 4 | 0.85 | 1.022 |
| 16 | 0.68 | 0.848 |
| 21 | 0.91 | 1.217 |
| 26 | 1.15 | 1.783 |
| 29 | 1.00 | 1.435 |
| 34 | 1.18 | 1.826 |
| 35 | 0.95 | 1.283 |
| 42 | 1.23 | 1.870 |

**Mean ± SD = (1.35 ± 0.37) µM**

*Gel retardation assay of SLE IgG with native and peroxynitrite–modified H2A histone*

Gel retardation assay was employed to visualize and confirm the interaction of native and 100 μM peroxynitrite–modified H2A histone with IgG antibodies isolated from SLE sera. A constant amount of native and modified–histone was incubated with increasing concentrations of SLE IgG for 2 hr at 37°C and overnight at 4°C. The antigen-antibody complex was then electrophoresed on 10% SDS-polyacrylamide gel for 4 hr at 80 V and stained with silver nitrate. Although high molecular mass immune complex with retarded mobility was observed with both native and peroxynitrite–modified H2A (Fig. 46 **a** and **b**), but in case of peroxynitrite–modified H2A histone we observed increasing number of retarded bands with increase in amount of SLE IgG. This difference may be due to formation of more than one type of immune complex of different mass in case of peroxynitrite–modified H2A. The free antigen showed proportional decrease of intensity.

## Studies on rheumatoid arthritis patients

*Direct binding ELISA of rheumatoid arthritis sera with native DNA, native H2A histone and 100 μM peroxynitrite-modified H2A*

All 50 rheumatoid arthritis samples included in this study were pre-tested for rheumatoid factor and found to be positive. Age- and sex- matched sera from apparently healthy individuals served as control. The sera were diluted 1:100 and subjected to direct binding ELISA on microtitre wells separately coated with equal amounts of native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A histone (Fig. 47–49). Sixteen RA sera (32%), $ labeled, showed higher binding with peroxynitrite–modified H2A histone compared to native DNA or H2A histone. No appreciable binding was observed with the pooled sera of normal subjects. These observations are important in view of the immunogenic nature of peroxynitrite–modified H2A histone and reported incidence of hypernitrotyrosinemia in good number of rheumatoid arthritis patients.

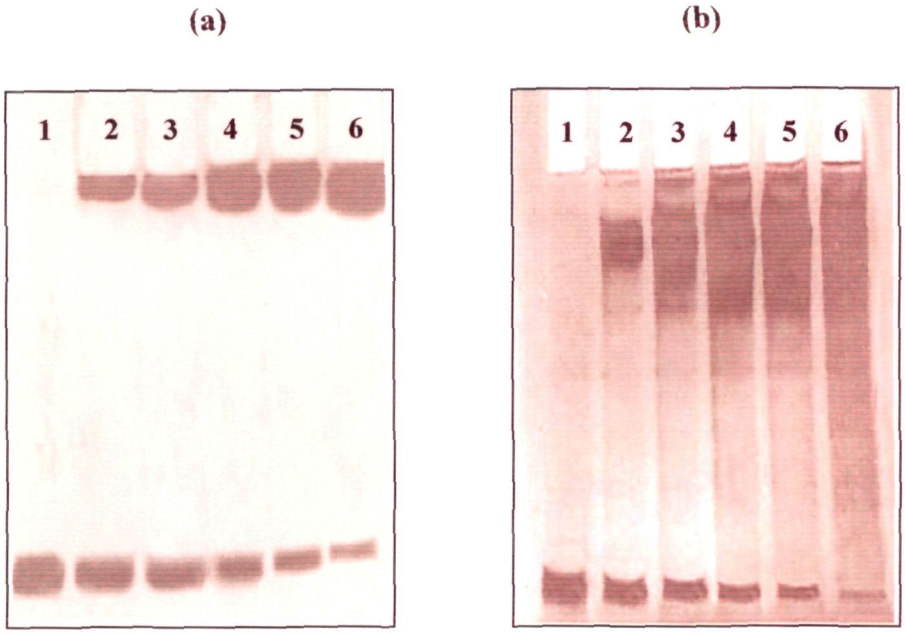



**Fig. 46** Gel retardation assay of a SLE IgG with native and 100 μM peroxynitrite–modified H2A histone in SDS-polyacrylamide gel. Electrophoresis was performed on 10% SDS–polyacrylamide gel for 4 hrs at 80 Volts.

**(a)** Native H2A (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated for 2 hrs at 37°C and overnight at 4°C.

**(b)** 100 μM peroxynitrite–modified H2A (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated under identical conditions.

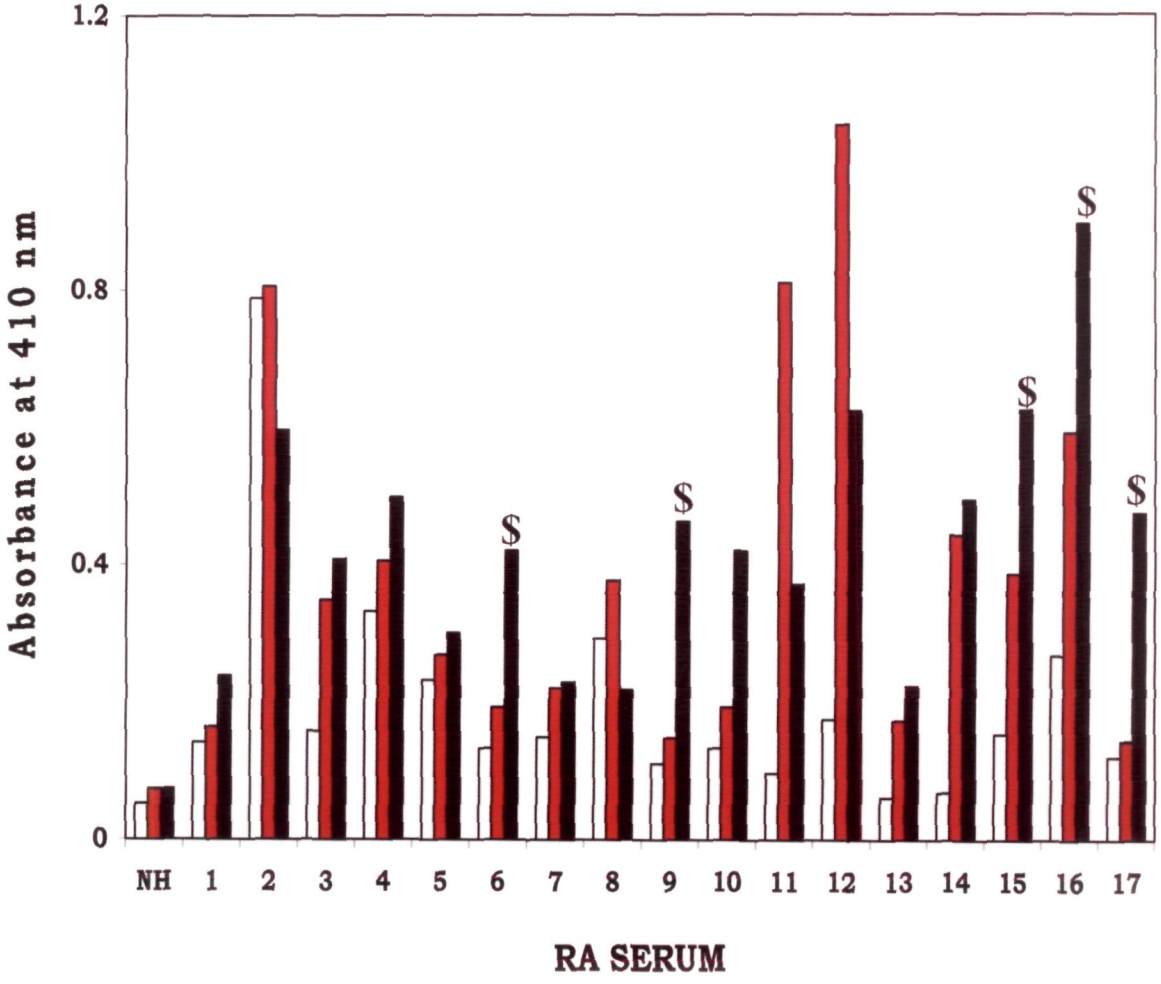



**Fig. 47** Binding of 1:100 diluted RA sera to native DNA (—■—), native H2A histone (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

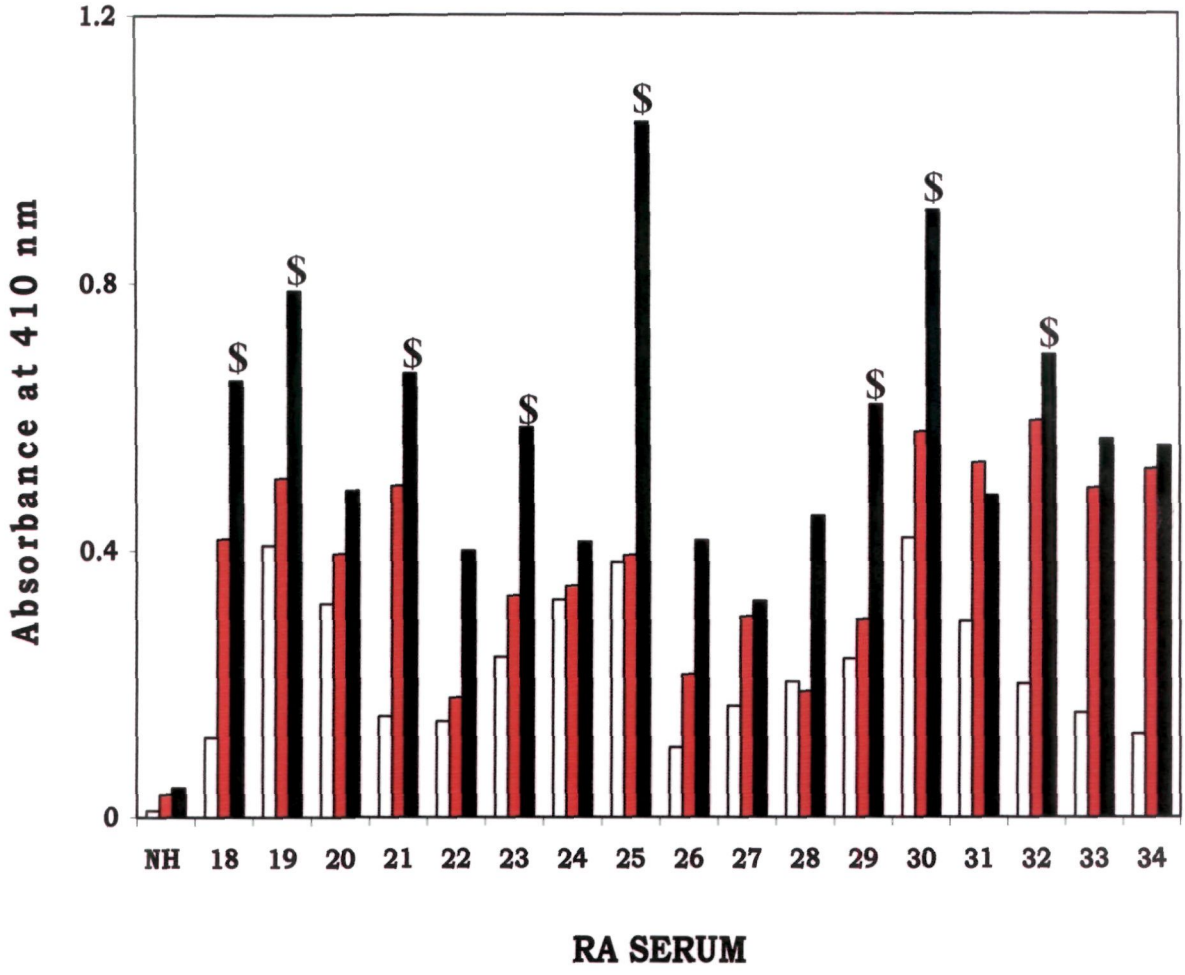



**Fig. 48** Binding of 1:100 diluted RA sera to native DNA (—■—), native H2A histone (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

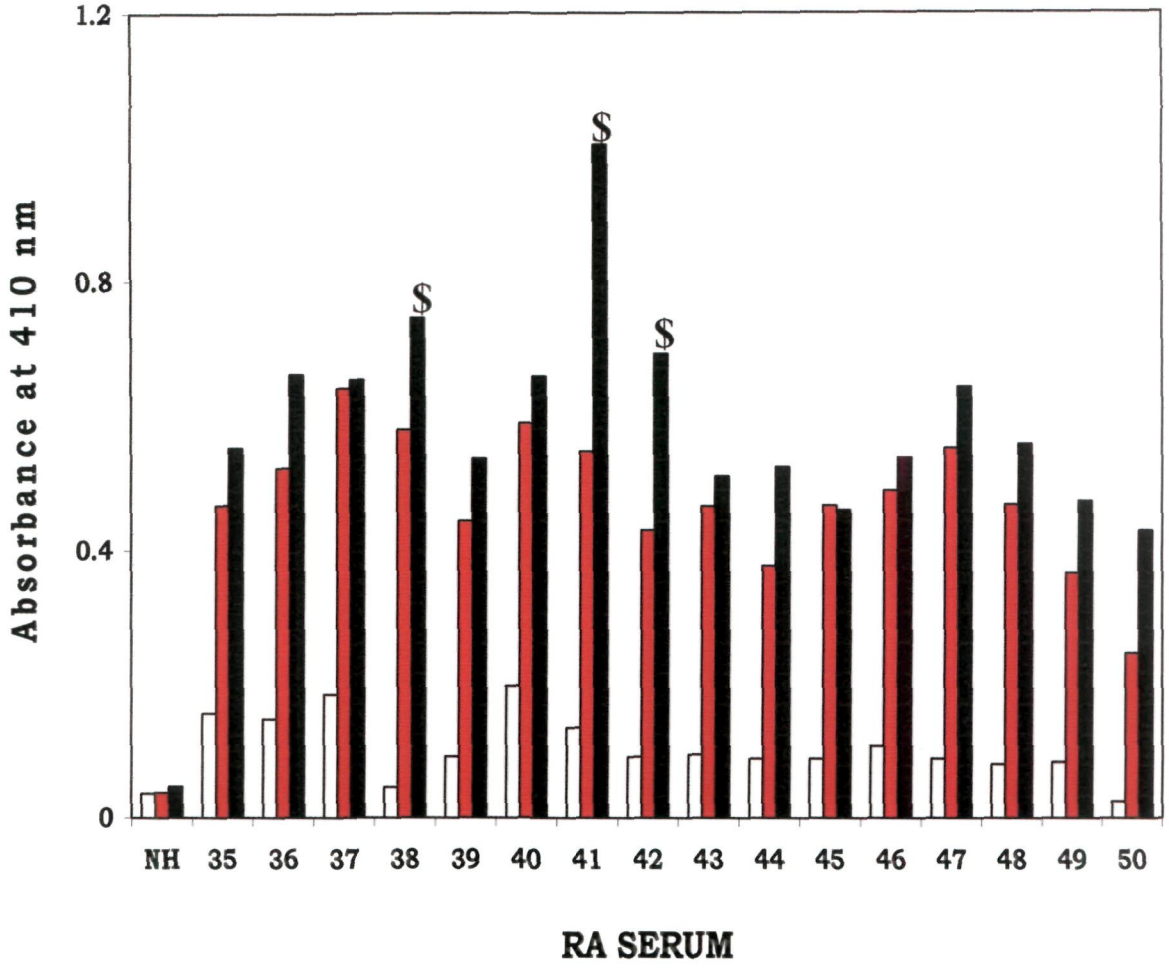



**Fig. 49** Binding of 1:100 diluted RA sera to native DNA (—■—), native H2A histone (—□—) and 100 μM peroxynitrite–modified H2A (—■—). Pooled normal human sera (NH) showed negligible binding with coated antigens.

*Enzyme immunoassay of rheumatoid arthritis IgG with native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A*

IgG were purified from sixteen rheumatoid arthritis sera which showed higher binding with peroxynitrite–modified H2A histone in direct binding ELISA. The purified rheumatoid arthritis IgG were first subjected to direct binding assay on microtitre wells coated with 100 μM peroxynitrite–modified H2A. This helped us to calculate amount of IgG antibodies required for antigen saturation. Direct binding profile of one rheumatoid arthritis IgG has been shown in Fig. 50.

The fine antigenic specificity of RA IgG was evaluated using native DNA, native H2A and 100 μM peroxynitrite–modified H2A as inhibitors. Rheumatoid arthritis IgG was separately mixed with native DNA, native H2A and 100 μM peroxynitrite–modified H2A histone (0–20 μg/ml) and incubated. The resulting complex was coated on antigen coated wells instead of only serum/IgG. Interaction of inhibitors with various rheumatoid arthritis IgG has been represented as percent inhibition in antibody binding with coated antigen (Fig. 51-54). The peroxynitrite–modified H2A histone emerged as the most powerful inhibitor followed by native DNA and native H2A. The summary of inhibition studies has been compiled in Table 15. Inter-comparison of data by a statistical method yielded a *p*-value of <0.001 for peroxynitrite–modified H2A vs native DNA or native H2A histone. It shows that the data are statistically significant. The immunogenic nature of peroxynitrite–modified H2A and reported incidence of hypernitrotyrosinemia in rheumatoid arthritis patients suggests that peroxynitrite–induced modification of proteins in and around joints might lead to an autoimmune response in a sub-population of RA patients and immune complex formation may further complicate the biochemistry of joints.

*Determination of protein carbonyl in rheumatoid arthritis sera*

Carbonyl contents in randomly selected 12 rheumatoid arthritis sera (no. 16–19, 21, 25, 30, 32, 36, 38, 41 and 42) and 12 normal human sera were determined. The average carbonyl contents of rheumatoid arthritis sera was found to be 3.15 ± 0.21 nmole/mg protein compared to 1.95 ± 0.20 nmole/mg protein in healthy subjects (Fig. 55). The statistical analysis revealed that rheumatoid arthritis

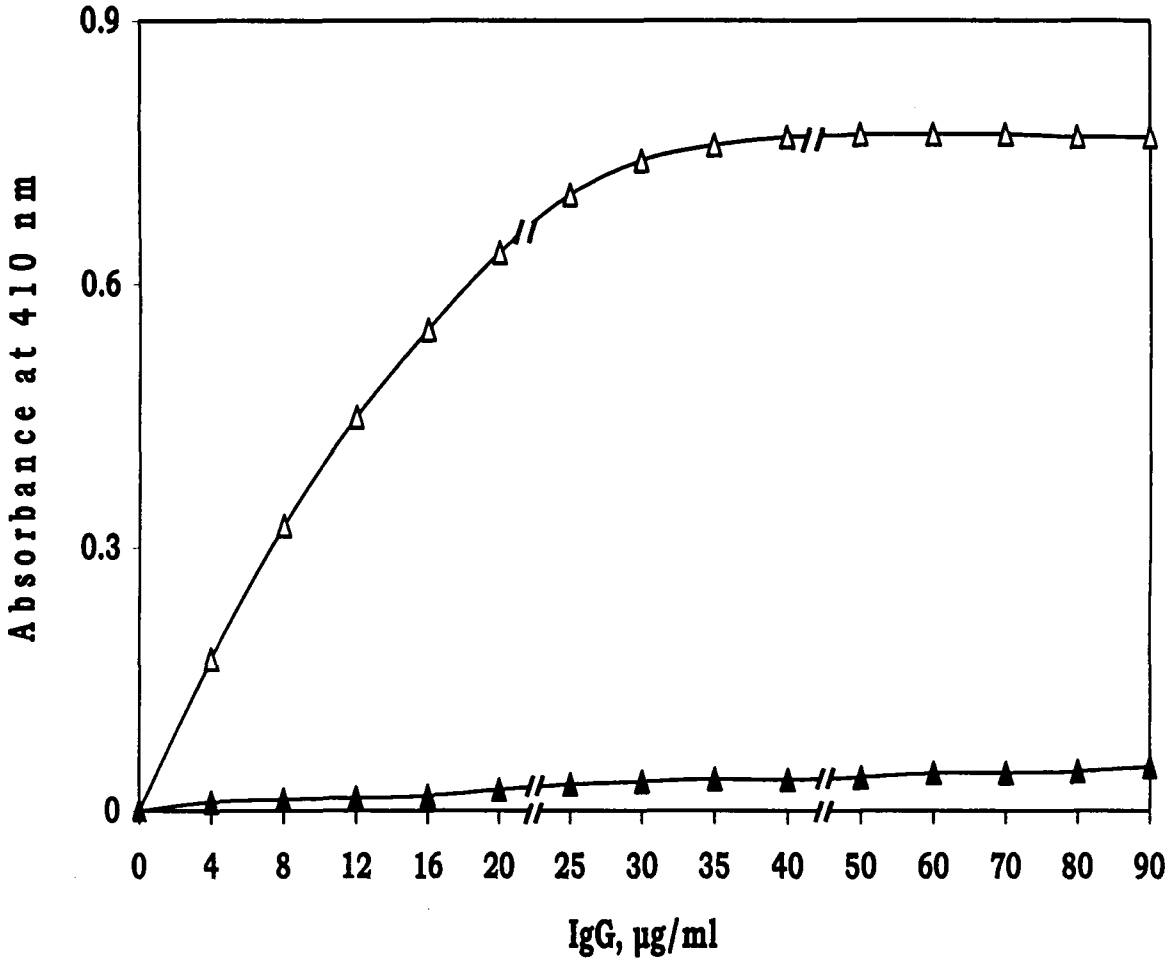



**Fig. 50** Direct binding ELISA of a purified RA IgG (—Δ—) and normal human IgG (—▲—) on microtitre wells coated with 100 µM peroxynitrite–modified H2A histone.

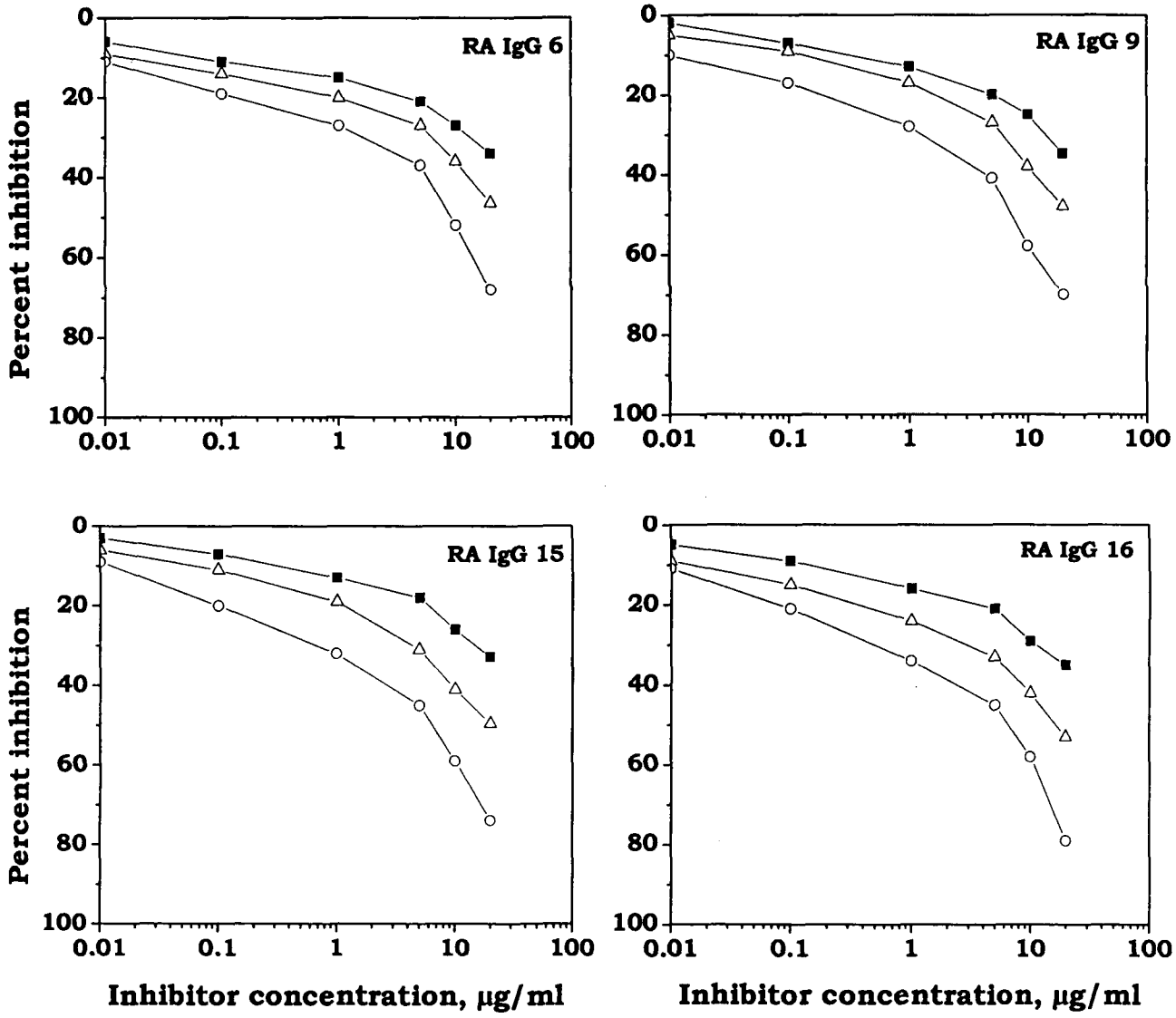



Fig. 51 Inhibition of RA IgG (isolated from sera **6, 9, 15** and **16**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 μM peroxynitrite–modified H2A histone (—O—). Microtitre wells were coated with native DNA (2.5 μg/ml).

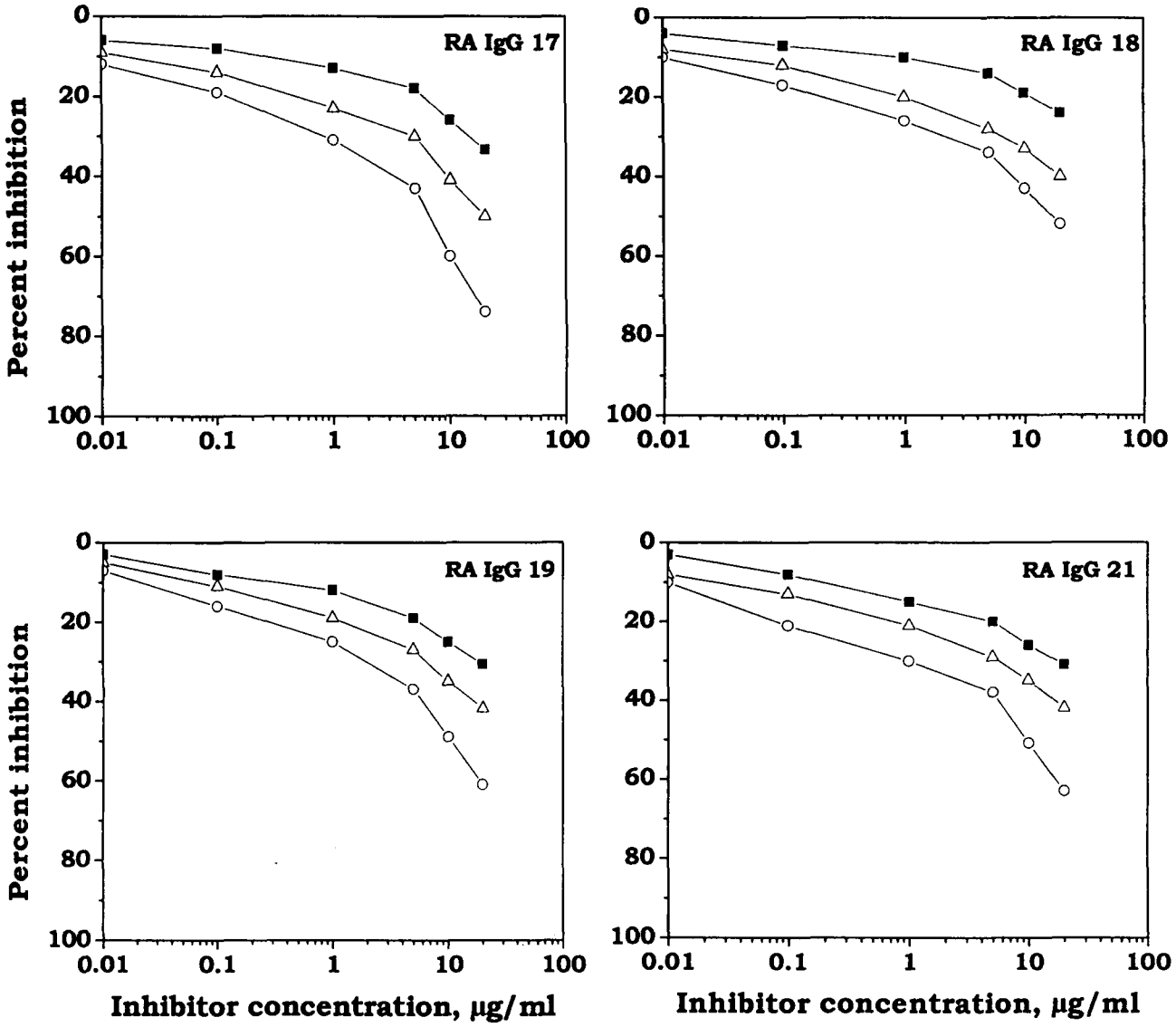



Fig. 52 Inhibition of RA IgG (isolated from sera **17, 18, 19** and **21**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite–modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

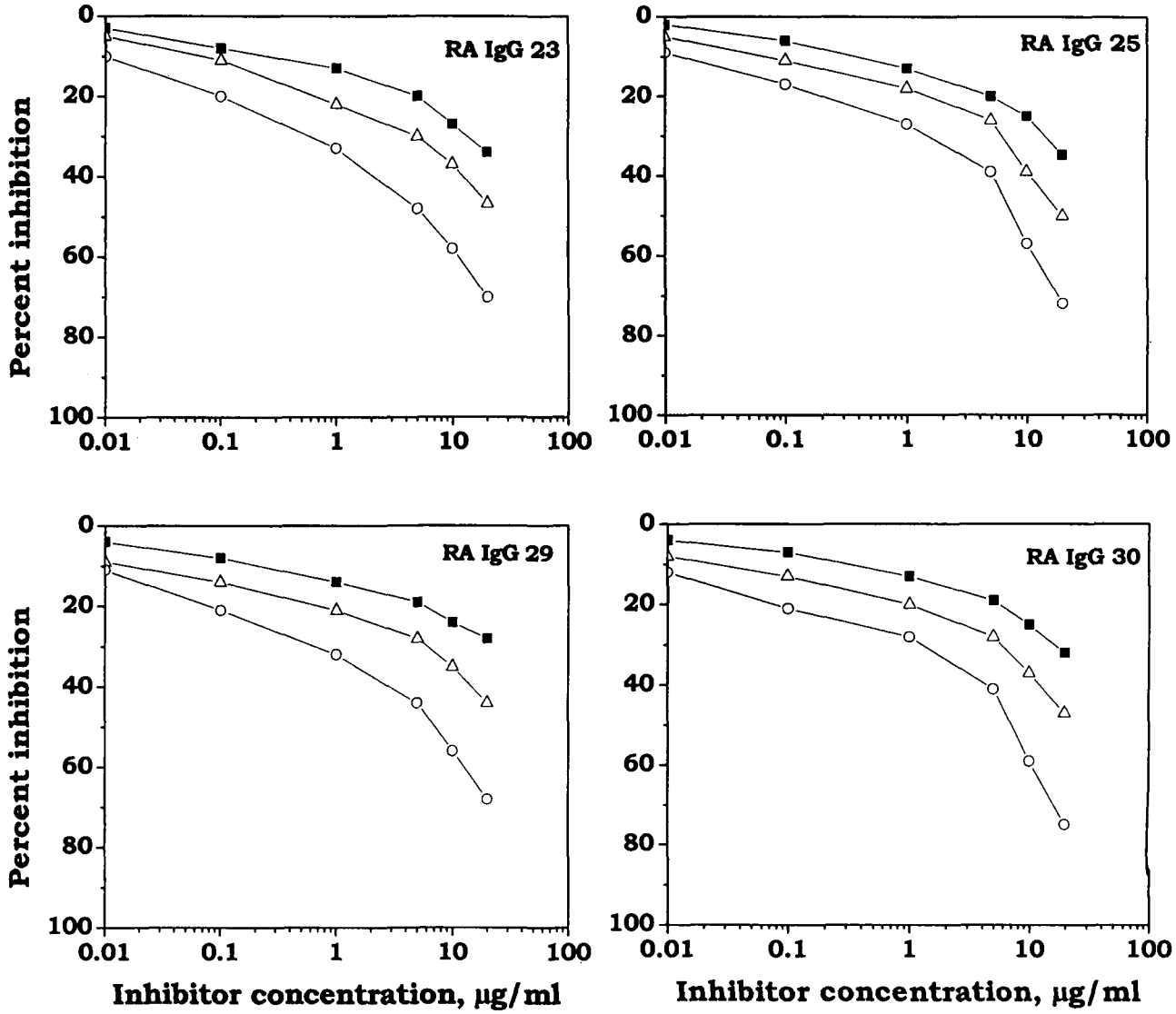



Fig. 53 Inhibition of RA IgG (isolated from sera **23, 25, 29** and **30**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite–modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

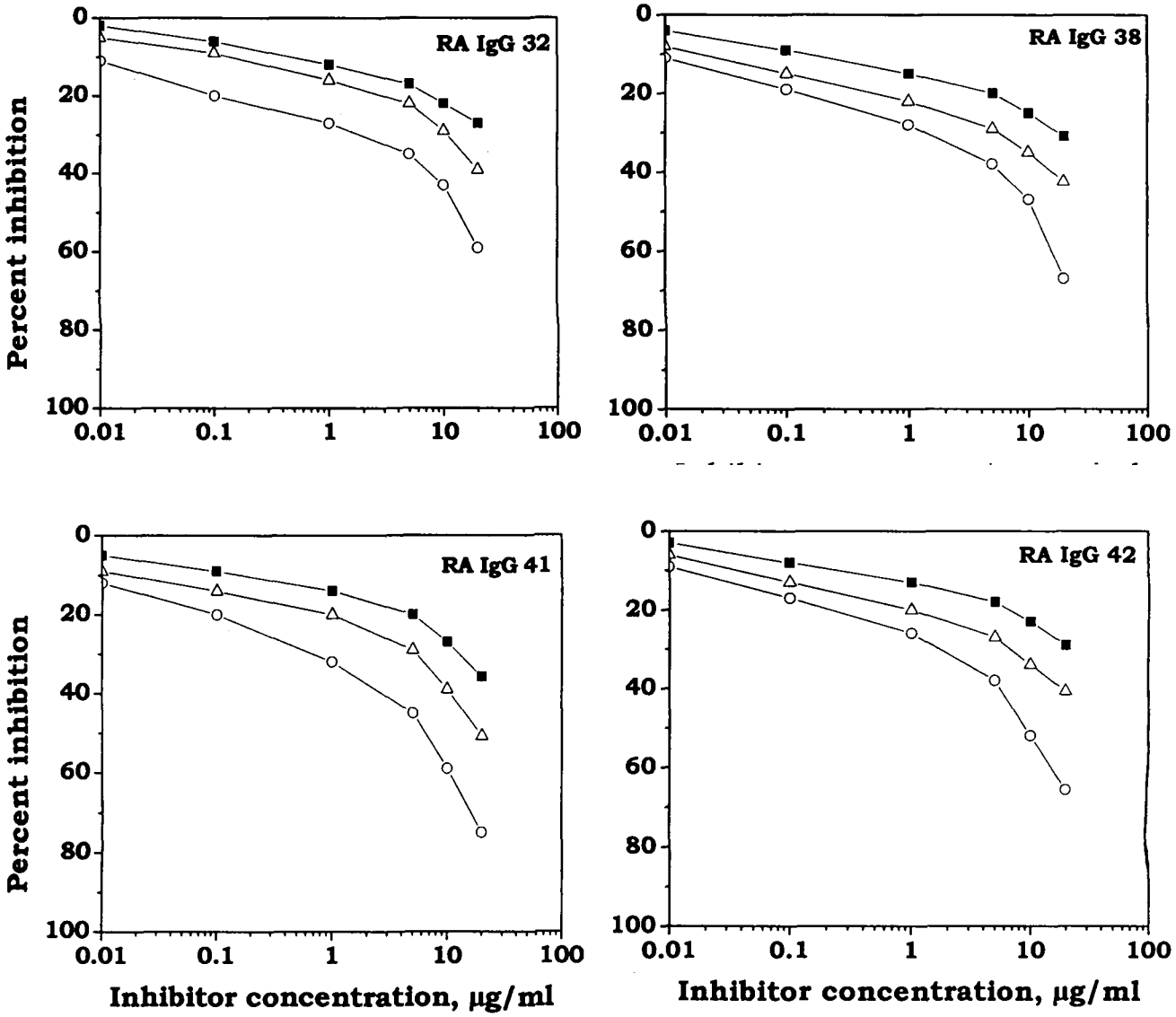



**Fig. 54** Inhibition of RA IgG (isolated from sera **32, 38, 41** and **42**) binding by native DNA (—Δ—), native H2A histone (—■—) and 100 µM peroxynitrite-modified H2A histone (—o—). Microtitre wells were coated with native DNA (2.5 µg/ml).

## Table 15

**[#] Inhibition of RA IgG binding by native DNA, native H2A histone and 100 μM peroxynitrite–modified H2A**

| IgG | Maximum percent inhibition at 20 μg/ml | | |
|---|---|---|---|
| | nDNA | nH2A | peroxynitrite–modified H2A |
| 01 (6)[$] | 46.4 | 34.0 | 68.0 |
| 02 (9) | 48.0 | 34.8 | 70.0 |
| 03 (15) | 49.5 | 32.8 | 74.0 |
| 04 (16) | 53.0 | 35.0 | 79.0 |
| 05 (17) | 50.0 | 32.4 | 74.0 |
| 06 (18) | 40.0 | 24.0 | 59.0 |
| 07 (19) | 41.8 | 30.7 | 61.0 |
| 08 (21) | 42.0 | 31.0 | 63.0 |
| 09 (23) | 46.7 | 34.0 | 70.0 |
| 10 (25) | 50.0 | 34.8 | 72.0 |
| 11 (29) | 44.0 | 28.0 | 68.0 |
| 12 (30) | 47.0 | 32.0 | 75.0 |
| 13 (32) | 39.0 | 27.0 | 59.0 |
| 14 (38) | 42.5 | 31.0 | 67.0 |
| 15 (41) | 50.8 | 35.8 | 75.0 |
| 16 (42) | 40.7 | 29.0 | 65.7 |
| **Mean ± S.D.** | **(45.71 ± 4.35)** | **(31.64 ± 3.30)** | **(68.73 ± 6.05)** |

[#] Microtitre plates were coated with native DNA.

[$] Numbers in parentheses show the corresponding RA serum number in Fig. 47–49 that was used to isolate IgG.

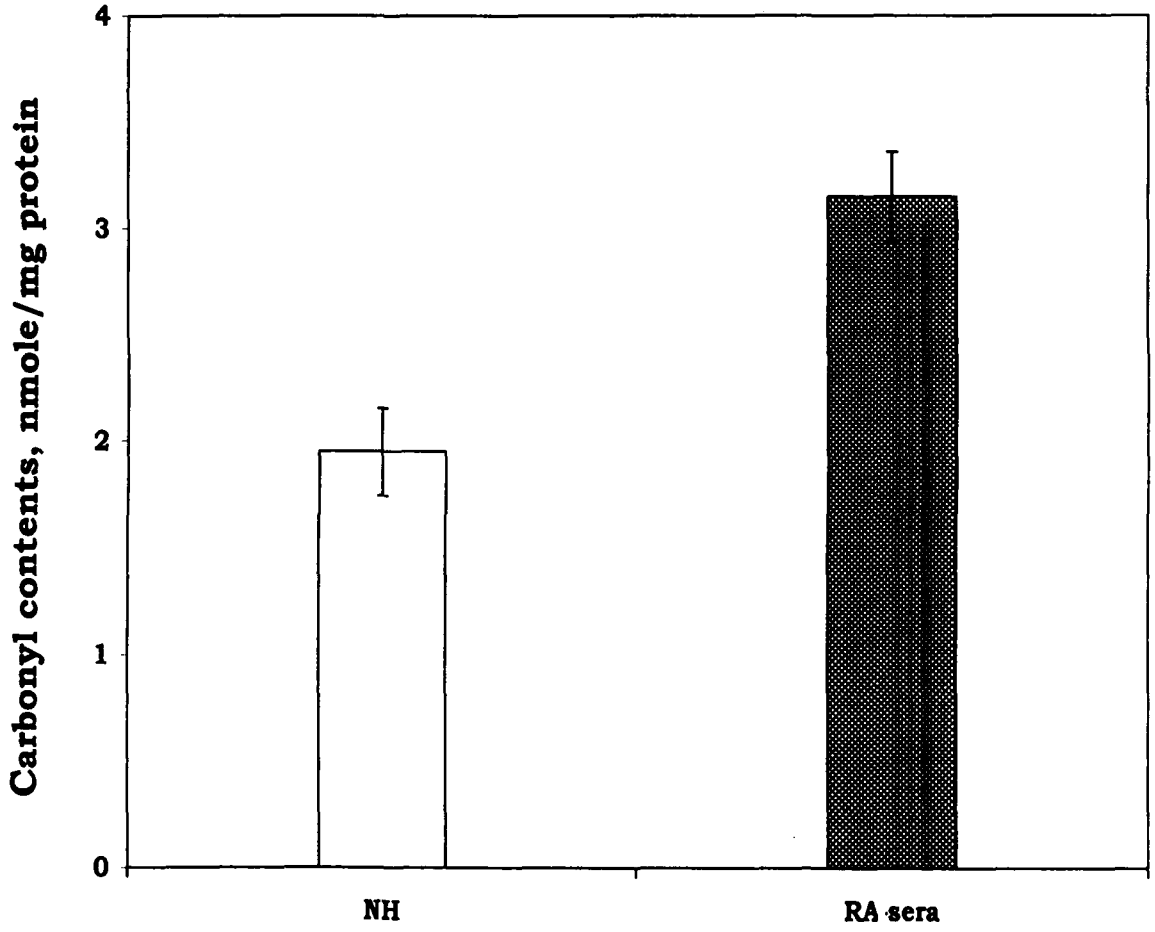



Fig. 55 Level of carbonyls in rheumatoid arthritis and normal human sera. Each histogram represents the mean ± S.D. of 12 samples.

samples had significantly higher content of carbonyls ($p < 0.05$) compared to sera of healthy subjects.

*Determination of nitrotyrosine in rheumatoid arthritis sera*

First of all, a fresh control was prepared by mixing equal amount of ten serum samples of apparently normal human subjects. Fig. 56 represents the HPLC chromatogram of pooled control serum. Nitrotyrosine peak (corresponding to ~12.25 min retention time) was not seen in the control serum. Results of HPLC analysis of 10 rheumatoid arthritis samples are given in Fig. 57-61. Nitrotyrosine was detected in all samples because we observed peaks matching with the retention time of standard 3-nitrotyrosine. Amount of 3-nitrotyrosine in rheumatoid arthritis sera was calculated from the calibration curve of standard 3-nitrotyrosine and the data are presented in Table 16.

*Gel retardation assay of rheumatoid arthritis IgG with native and peroxynitrite–modified H2A histone*

Once again we employed gel retardation assay to visualize and confirm the true interaction of IgG, purified from RA patients' sera with native and 100 μM peroxynitrite–modified H2A histone. A constant amount of native and modified–histone was mixed with increasing concentrations of RA IgG and incubated. The resulting complex was then electrophoresed on 10% SDS-polyacrylamide gel for 4 hr at 80 V and stained with silver nitrate. Results shown in Fig. 62 demonstrate that IgG antibodies purified from RA serum is recognizing both old (present on native H2A) and new epitopes (appeared after peroxynitrite modification). In case of native H2A, the increasing amount of IgG antibodies appears to have initially produced immune complex of almost similar size which later marginally increased in size as reflected by corresponding decrease in amount of unbound antigen. Incubation of RA IgG antibodies with peroxynitrite–modified histone generated immune complex of different size and mobility and the unbound antigen showed matching decrease. In this case, it seems that both old and new epitopes co-existing on peroxynitrite–modified histones are being recognized by IgG antibodies, and thus immune complex of large size have been observed.

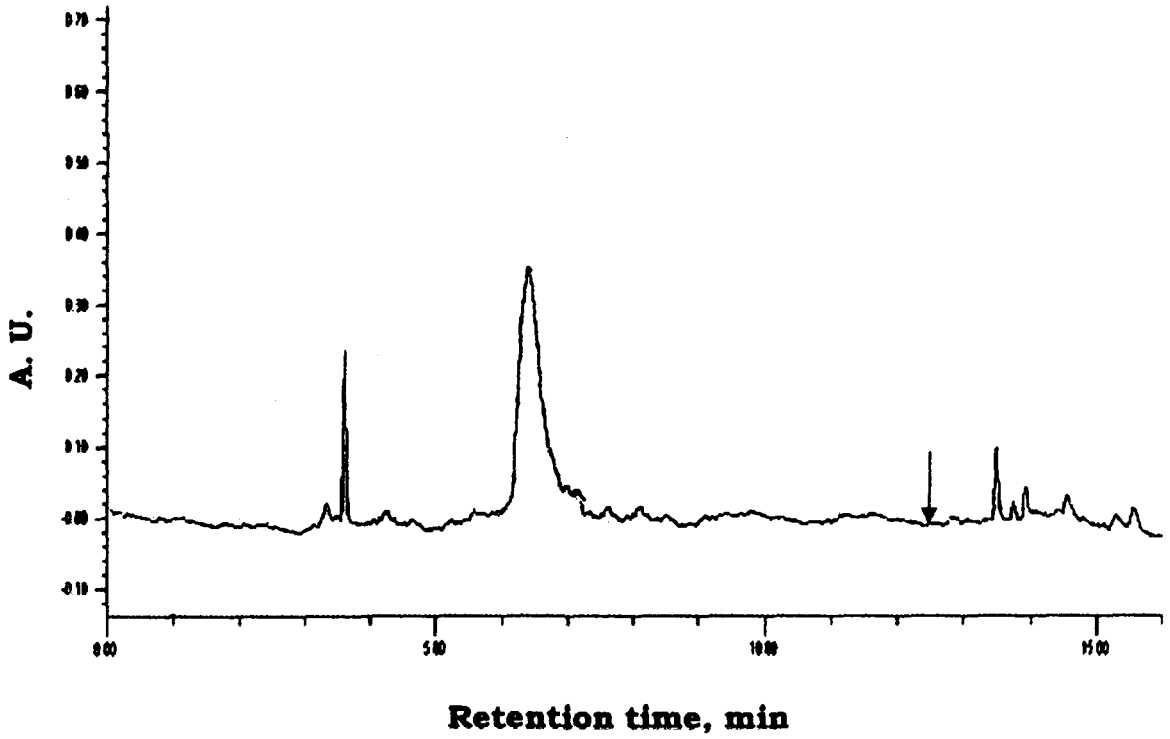



**Fig. 56** HPLC profile of the pooled control serum. The down head arrow points out the absence of nitrotyrosine in the control which otherwise appears at this retention time.

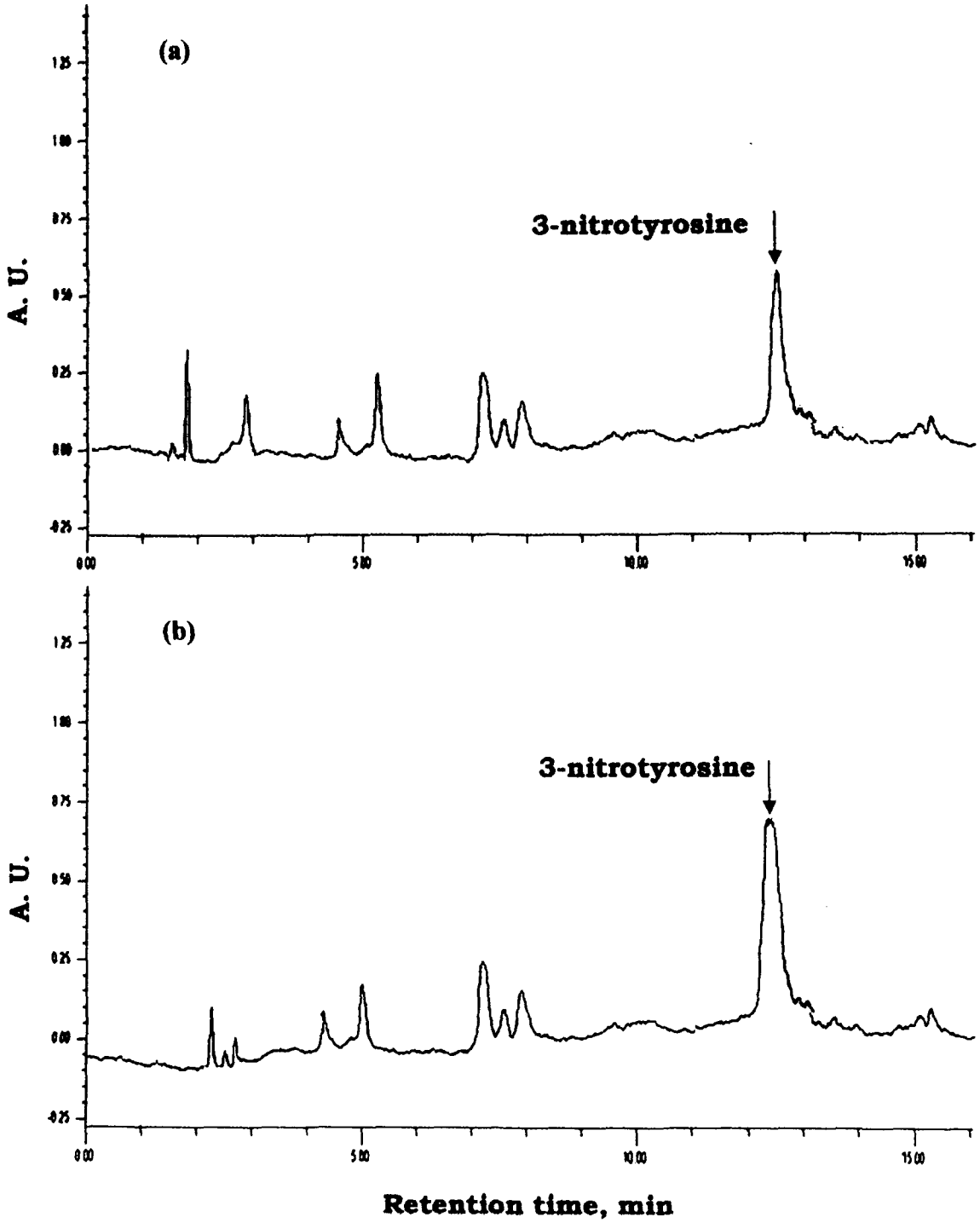



**Fig. 57** HPLC profile of RA serum sample number 4 (**a**), and number 6 (**b**). The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

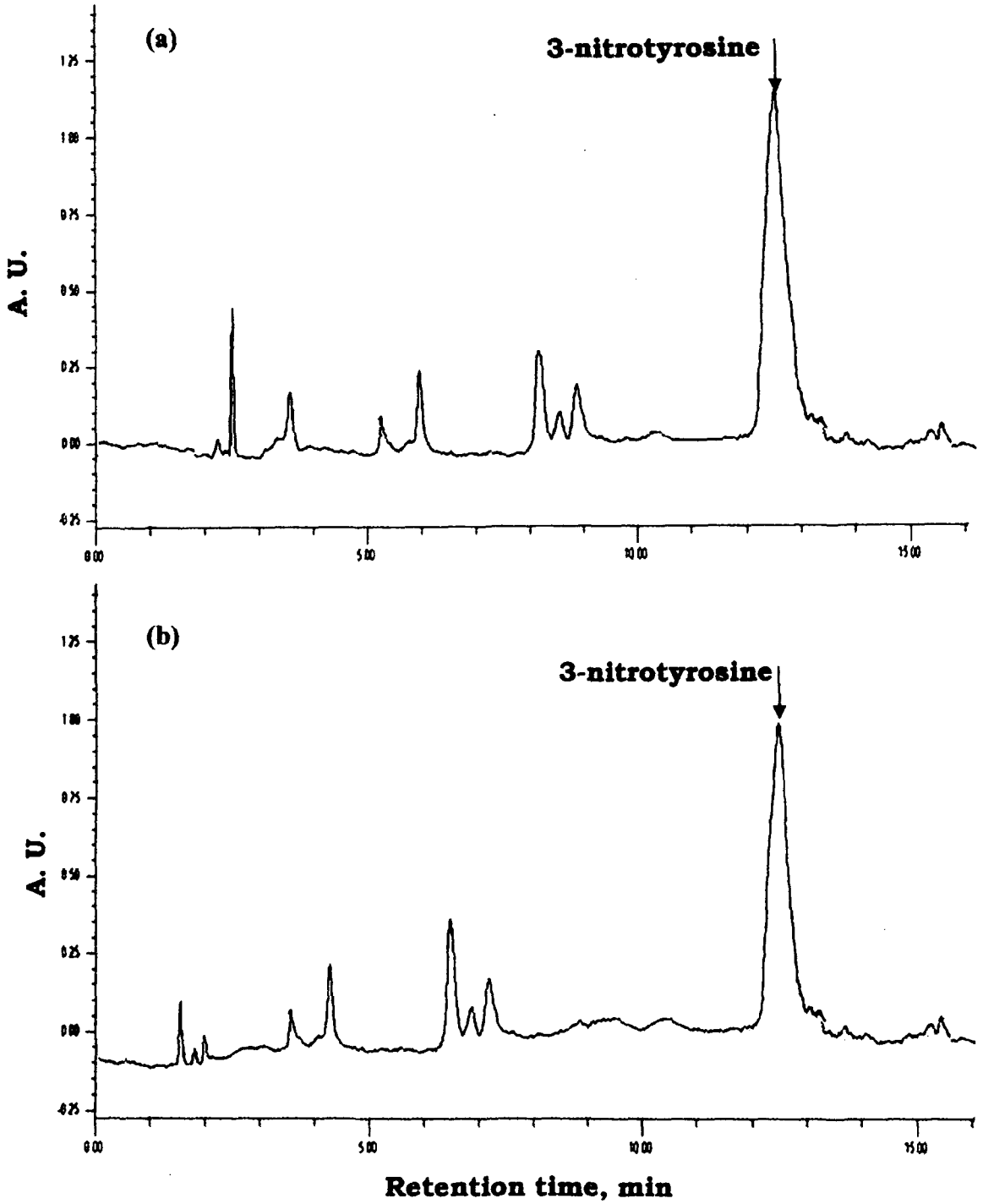



**Fig. 58** HPLC profile of RA serum sample number 14 **(a)**, and number 16 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

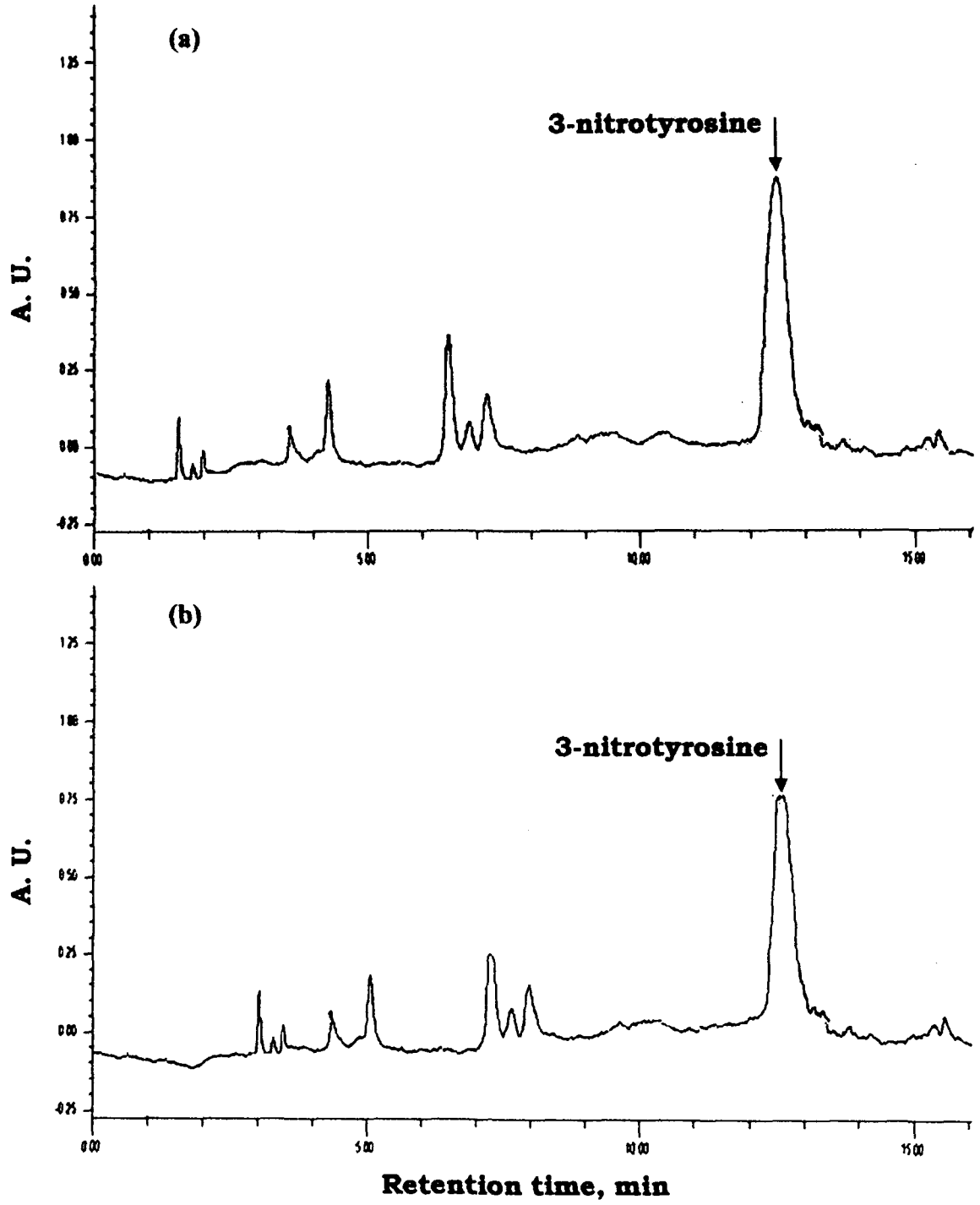



**Fig. 59** HPLC profile of RA serum sample number 17 **(a)**, and number 23 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

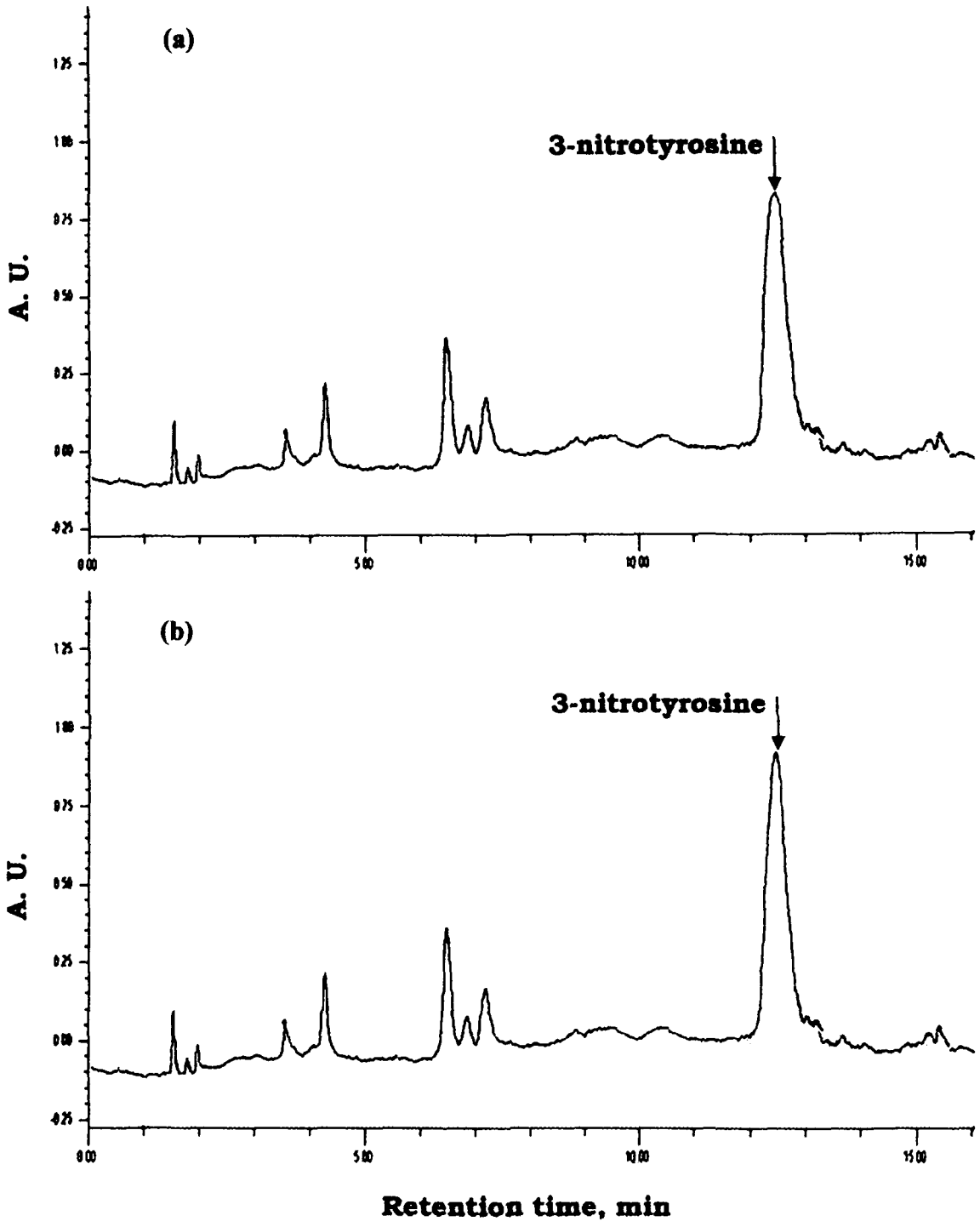



**Fig. 60** HPLC profile of RA serum sample number 25 **(a)**, and number 30 **(b)**. The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

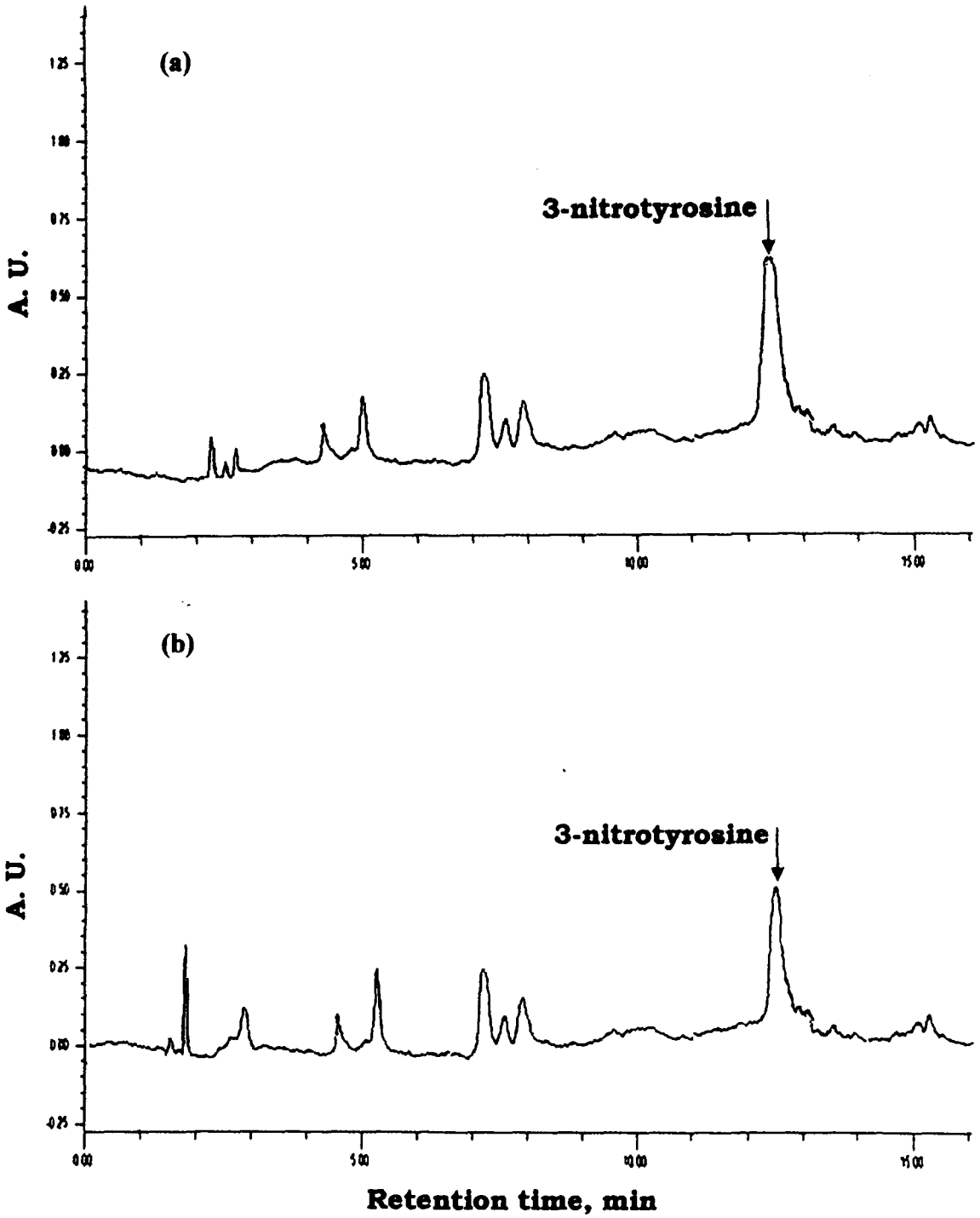



**Fig. 61** HPLC profile of RA serum sample number 38 (**a**), and number 42 (**b**). The figures have well defined peaks matching the retention time of standard 3-nitrotyrosine.

## Table 16

**Quantitative estimation of nitrotyrosine in RA sera by HPLC**

| Rheumatoid arthritis serum sample number | Absorbance at 274 nm | µM of niytotyrosine |
|---|---|---|
| 4 | 0.56 | 0.674 |
| 6 | 0.67 | 0.815 |
| 14 | 1.12 | 1.739 |
| 16 | 0.98 | 1.392 |
| 17 | 0.87 | 1.065 |
| 23 | 0.75 | 0.913 |
| 25 | 0.81 | 0.978 |
| 30 | 0.93 | 1.348 |
| 38 | 0.62 | 0.772 |
| 42 | 0.52 | 0.652 |

**Mean ± SD = (1.035 ± 0.35) µM**

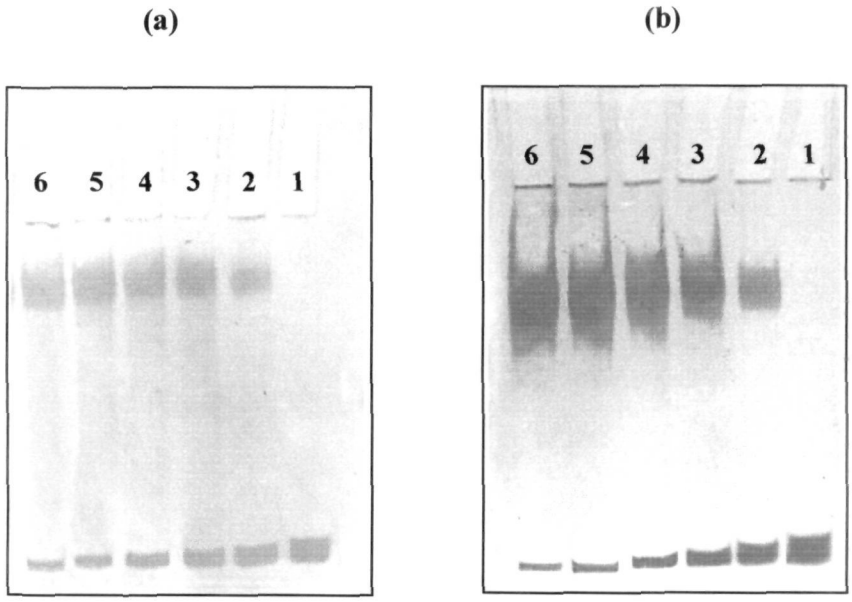



**Fig. 62** Gel retardation assay of an IgG isolated from rheumatoid arthritis serum with native and peroxynitrite–modified H2A in SDS–polyacrylamide gel. Electrophoresis was performed on 10% SDS–polyacrylamide gel for 4 hrs at 80 Volts.

**(a)** Native H2A (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated for 2 hrs at 37°C and overnight at 4°C.

**(b)** 100 μM peroxynitrite–modified H2A (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of anti-peroxynitrite–modified H2A IgG (lanes 2–6) and incubated under identical conditions.

# Discussion

Nitric oxide ($NO^{\bullet}$) is an endothelium derived gaseous molecule. Chemically, it is a free radical but participates in numerous physiological processes; neurotransmission, blood pressure regulation, defense mechanism, smooth muscle relaxation and immunity (Archer, 1993; Alderton *et al.*, 2001; Bergendi *et al.*, 1999; Forstermann *et al.*, 1998). Action of nitric oxide synthase on arginine produces $NO^{\bullet}$ and citrulline (Ghafourifar and Cadenas, 2005). Furthermore, ubisemiquinone formed at the ubiquinol oxidation center of the cytochrome $bc_1$ complex is one of two sources of electrons for superoxide formation in mitochondria (Dröse and Brandt, 2008). Cells of the immune system produce both superoxide anion and nitric oxide during oxidative burst triggered by inflammation. In such situations nitric oxide and superoxide ion may combine to produce peroxynitrite ($ONOO^{-}$) which causes oxidation and nitration in biomolecules (Carr *et al.*, 2000). Peroxynitrite reactivity towards proteins, lipids, amino acids, nucleic acids etc. account for its complex chemistry (Ducroig *et al.*, 1999). Because of its oxidizing nature it can directly react with electron rich groups; such as sulfhydryl (Redi *et al.*, 1991b), ion sulfer centres (Castro *et al.*, 1994), zinc-thiolates (Crow *et al.*, 1995) and active site sulfhydryl in enzymes (Takakura, *et al.*, 1999). Peroxynitrite can also produce novel products such as nitrotyrosine, nitrotryptophan and nitrated lipids. Thus, this intermediate can produce oxidative and nitrosative damage in biomolecules, which in turn may lead to autoimmune- and age-related diseases (Drew and Leeuwenburg, 2002; Oates and Gilkeson, 2006). Systemic lupus erythematosus (SLE) is an autoimmune multifactorial disease of unknown etiology. It is characterized by presence of circulating and tissue-fixed autoantibodies to a variety of self and non-self antigens including native DNA, histones, non-histone protein antigens and protein-nucleic acid complex. Like SLE, the trigger for rheumatoid arthritis (RA) remains obscure. Inflammation of the synovial membrane of diarthrodial joints along with rheumatoid factor characterizes RA. Rheumatoid factor is an autoantibody that reacts with Fc portion of the IgG and serves as an immunological marker in RA. Other autoantibodies have also been reported in RA but they lack specificity.

Histones are small cationic proteins which bind DNA. They are weak immunogen probably due to their conserved nature. Histones are major constituent

of cells' chromatin and remain confine to nucleus. However, after apoptosis they may appear in circulation as nucleosomes. Incidence of autoantibodies against H1, H2A, H2B, H3 and H4 histones in sera of SLE patients are 60, 53, 48, 36 and 29.5% respectively (Ghedira *et al.*, 2006).

In this study, aqueous solutions of H1, H2A, H2B and H3 histones were exposed to laboratory synthesized peroxynitrite and the structural changes have been analyzed by physico-chemical techniques. Inhibition of nitration and oxidation by reactive nitrogen- and reactive oxygen species inhibitors confirmed the generation of peroxynitrite.

Native structures of H1, H2A, H2B and H3 histones showed a single peak having maximum absorbance at 276 nm. With increasing concentration of peroxynitrite the 276 nm peaks of histones showed hyperchromicity. This might be due to increasing nitration of tyrosine residues which ultimately enhances the molar absorptivity compared to native histones (Sokolorsky *et al.*, 1966). Furthermore, appearance of a new peak in peroxynitrite–modified histones appears to be due to generation of nitrotyrosine because a standard solution of 3-nitrotyrosine had matching profile. Moreover, appearance of yellow color in peroxynitrite-modified histones strongly substantiates the formation of nitrotyrosine which gave absorption peak at 420 nm. HPLC analyses of peroxynitrite-modified histones confirmed the presence of nitrotyrosine in samples because one of the peaks had almost similar matching retention time as shown by standard 3-nitrotyrosine.

Chromophoric groups like tryptophan, tyrosine and phenylalanine contribute significantly to intrinsic fluorescence of proteins. But in case of histones only tyrosine residues are involved in intrinsic fluorescence because histones lack tryptophan, and phenylalanine is a weak chromophore as well as fluorophore. The fluorophoric property of tyrosine was taken into account in order to analyze the effect of peroxynitrite-mediated nitration of tyrosine. The modified histone samples exhibited fluorescence quenching compared to native histones. The quenching appears to be the consequence of introduction of nitro group in tyrosine's phenolic ring (Uversky *et al.*, 2005).

Native unfolded proteins with their unique combination of ordered structure (little or nil) under physiological conditions represent a distinct subdivision of the protein kingdom (Uversky *et al.*, 2000). A combination of overall low hydrophobicity and large net charge is a characteristic property of histone proteins. Peroxynitrite–modified histones' partially folded structures were characterized by availability of exposed hydrophobic clusters to which ANS had showed binding. This interaction resulted in a considerable increase in the ANS fluorescence intensity and a pronounced blue shift of emission maxima in comparison to native histones. Moreover, the large blue shift in the ANS fluorescence maxima reflects nitration induced transformation in histones from the natively unfolded state to a partially folded structure. Native histones are found in unfolded state due to their low hydrophobicity but as a result of peroxynitrite modification the positive charges are neutralized and this favors exposure of more hydrophobic clusters *inter alia* binding of ANS.

Histones structural modification was further confirmed by far UV-CD spectroscopy. The far UV-CD spectra of native histones under our experimental conditions were found to be typical of significantly unfolded proteins. Upon modification with peroxynitrite, the histones showed increase in ordered structure compared to native histones. This was manifested by an increase in the negative ellipticity around 222 nm which is taken as the measure of ordered secondary structures (Munishkina *et al.*, 2004). The above changes were further substantiated by FT-IR spectroscopy. The main advantage of FT-IR in comparison to CD is that the FT-IR is much more sensitive to β-structures (Oberg and Fink, 1998). We recorded the FT-IR (amide 1 region) spectra of native and 100 μM peroxynitrite–modified histones. The most prominent change that we observed was the decrease of band intensity at higher wavenumber which correspond to the disordered conformation accompanied by the appearance of a new band in the vicinity of lower wavenumber, which corresponds to ordered or β-sheet structure. Thus the results of CD and FT-IR may be considered as proof that after modification with peroxynitrite histones show more ordered structures than native histones.

Peroxynitrite–induced structural changes in histone were visualized in gel. The results threw further light on the substantial structural changes that occurred in

histones upon peroxynitrite modification. Formation of inter– and intra– molecular cross-links does appear during nitration of histones with peroxynitrite (Glazer, 1976) and cross-linking & oligomerization have been reported during nitration of several proteins (Lundblad and Noyes, 1984). A marked retardation in the mobility of peroxynitrite–modified histones compared to corresponding native forms (except for H2B histone) suggest generation of high molecular weight species due to cross-linking. For H2B, the peroxynitrite–modified form did not show any retardation. However, we observed thickness of bands and that may be due to binding of more silver ions to additional aromatic amino acid residues that might have been exposed after treatment with peroxynitrite.

Generation of tyrosyl radical by peroxynitrite and its subsequent reaction with another tyrosyl radical (on same or different molecule) may form O-O'–dityrosine covalent cross links. To provide additional support to cross-linking mechanism, we estimated dityrosine content in peroxynitrite–modified histones by absorption measurements (Ischiropoulos and Al-Mehdi, 2005). The data suggest formation of more dityrosine in H2A, H2B and H3 as compared to H1 histone and therefore cross-linking and dityrosine may be inter-connected. Melting studies demonstrated that interaction of histones with peroxynitrite resulted in stability of peroxynitrite–modified histones compared to native histones because melting temperature was raised. Here, we would like to point out that our results have indicated that nitration leads to formation of partially folded structure from natively unfolded form and therefore rise in melting temperature is fully justified.

Carbonyl content is the most commonly used marker of protein oxidation (Shacter, 2000) and accumulation of protein carbonyl has been reported in many human diseases (Beal, 2002). During our modification studies, carbonyl content of histones increased upon treatment with different doses of peroxynitrite. Since peroxynitrite is both an oxidant and a nitrating agent, the increase in carbonyl content may be attributed directly to oxidative action of peroxynitrite.

Histones are conserved proteins and weak immunogens. However, these proteins show strong immunogenicity after acetylation and nitration (Muller *et al.,* 1987). It has been suggested that alteration in amino acids structure or sequence may generate neo-epitopes on self proteins leading to autoaggeressive

immune attack. Furthermore, autologous proteins may also become immunogenic if they are structurally modified. These modifications may generate or mask antigenic epitopes and that may stimulate relevant B-cells and /or T-cells leading to breakdown or bypass of tolerance.

In rabbits, the peroxynitrite–modified H2A histone was found to be a potent immunogen as revealed by high titre antibodies. The induced antibodies were immunogen specific but showed cross-reaction with non-modified H2A histone. Hence, immunization with peroxynityrite–modified H2A produced polyspecific antibodies which could recognize both old and neo-epitopes or altogether there are two types of antibodies, one recognizing nitrated neo-epitopes and another binding exclusively with old epitopes. Antigenic specificity of purified immune IgG was confirmed by inhibition ELISA. More than 90 percent inhibition in binding of anti-peroxynitrite–modified H2A histone antibodies with immunogen (as inhibitor) is a clear evidence of specificity.

Cross-reaction studies on anti-peroxynitrite–modified H2A histone antibodies with an array of nitrated proteins suggest recognition of common antigenic epitope on nitrated proteins. And that common epitope may be nitrotyrosine. Furthermore, we found that induced antibodies showed binding with H2B, H1, H3 histones, HSA, proteins rich in tyrosine residues and nucleic acids. Visual detection of interaction between immune IgG and the immunogen was facilitated by gel retardation assay. The results reiterated specificity of induced antibodies towards immunogen. Anti-peroxynitrite–modified H2A histone antibodies did not show appreciable binding with the native H2A histone in gel. It may be said that induced antibodies are predominantly directed against the antigenic determinants generated as a consequence of peroxynitrite modification of H2A histone.

Autoantibodies against intracellular proteins and nucleic acids are serological hallmark of the systemic rheumatic disease such as SLE, Progressive systemic sclerosis (PSS), Sjogren's syndrome (SS), Mixed connective tissue diseases (MCTD) and Polymyositis (PM) (Shoenfeld *et al.*, 1989). Each of these diseases is identified by the unique autoantibodies. Antibodies to dsDNA serve as an immunological marker for the diagnosis of SLE (Su *et al.*, 2007). These

autoimmune diseases are associated with multiple antinuclear antibody specificities, suggesting a role for both generalized as well as antigen-specific immune abnormalities in their etiology (Pisetsky, 1992; Herrmann *et al.*, 1995). The peroxynitrite derived species causes extensive damage to proteins and may render them immunogenic.

Despite decades of expansive research work on SLE, the exact nature of antigen stimulus for SLE initiation and progression remains unknown. We attempted to investigate the binding characteristics of naturally occurring SLE anti-DNA autoantibodies to native calf thymus DNA and native & peroxynitrite-modified-H2A histone so that some light could be thrown on the possible role of peroxynitrite-modified H2A histone in SLE. Sera of SLE patients, having high titre anti-DNA antibodies, showed preference for peroxynitrite–modified H2A histone compared to native H2A histone or native DNA. This suggests that peroxynitrite–modified H2A histone is an effective inhibitor of native DNA-anti-DNA antibody interaction. Band shift assay and inhibition ELISA with SLE IgG substantiated the preferential binding of peroxynitrite–modified H2A histone with SLE autoantibodies. It may be interpreted that nitration of tyrosine residues resulted in generation of neoantigen(s) with explicit immune response compared to native H2A histone. The body's immunosurveillance may prove ineffective if the generation of peroxynitrite is enhanced tremendously, as seen in chronic inflammation and in injured tissues. Once peroxynitrite level increases, the damage and nitration would be inevitable and the immunoregulatory network would be activated to deal with this alien nitration and modification. The subsequent production of autoantibodies would be natural step. Separation and quantitation of 3-nitrotyrosine in SLE sera were carried out by HPLC. The SLE sera showed high level of free 3-nitrotyrosine which was not present in control subjects. We observed that nitrotyrosine level in SLE sera had a good correlation with disease severity (Oates *et al.*, 1999). Thus, measurement of nitrotyrosine may be a useful way of determining peroxynitrite–mediated pathology and testing the effectiveness of therapeutic agents in preventing such diseases. Cellular metabolism and ionization radiation produces both reactive nitrogen & oxygen species. These radicals and their subsequent intermediates may react with cellular

macromolecules and induce a variety of chemical alterations leading to autoantibodies (Rasheed *et al.*, 2007). The oxidation of a protein typically results in increase in carbonyl contents. The increase in carbonyl has been attributed to oxidation of lysine, arginine, proline or other amino acid residues. In short, protein carbonyl groups are the biomarker of oxidative stress (Dalle-Dome *et al.*, 2003). In human plasma, all amino acids in protein are susceptible to oxidative modification by hydroxyl radical and hypochlorous acid (Levine *et al.*, 1994). In SLE, reactive nitrogen intermediates are over produced due to over expression of inducible nitric oxide synthase (iNOS) which may lead to tissue injury and increase carbonyl groups in a sub-group of SLE patients. The mechanism through which peroxynitrite can be pathogenic in the setting of SLE is through the generation of neo-epitopes on self antigens. Serum from SLE patients exhibited increased binding to peroxynitrite–modified H2A histone compared to native DNA or native H2A histone. This suggests that peroxynitrite modification of self antigens can generate neo-epitopes with increased binding affinity over native antigens.

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane of diarthrodial joints. Lack of a specific marker antibody is particularly true for rheumatoid arthritis. Rheumatoid factor (RF) is an autoantibody that reacts with the Fc portion of IgG and serves as one of immunological marker for this disease. A number of additional autoantibodies have been reported in RA patients including antibodies to filaggrin, citrullin, keratin, components of the spliceosome, anti-nuclear antibodies etc. (Pisetsky, 1992; Herrmann *et al.*, 1995). Nitrotyrosine has emerged as a stable end product and marker of inflammation and reactive nitrogen species production (Oates *et al.*, 1999). Sera of RA patients having high titre rheumatoid factor were analyzed for autoantibodies against native DNA and native & peroxynitrite–modified H2A histone. All sera of RA patients showed enhanced binding with peroxynitrite–modified H2A histone compared to native H2A histone or native DNA. Separation and quantitation of 3-nitrotyrosine in randomly selected RA sera were carried out by HPLC. All tested samples of rheumatoid arthritis had significantly higher level of nitrotyrosine compared to normal healthy subjects. Here also, we observed direct correlation between disease severity and nitrotyrosine level. Our results are in conformity with earlier

observation that peroxynitrite or its intermediates might be a mediator of joint damage in chronic inflammatory joint diseases because permeability increases in rheumatoid arthritis (Levick, 1981; Khokha *et al.,* 2003). This may encourage nitration of protein tyrosine followed by immune response to the neo-antigens. The present study demonstrates that autoantibodies against nitrotyrosine might be helpful in monitoring the progress of disease from mild to chronic stage and preventive measures, like controlling the excessive production of RNS, could be exercised in time. Furthermore, level of nitrotyrosine in combination of other clinical features of the RA might be a powerful diagnostic tool.

Finally, it may concluded that histones modified by laboratory synthesized peroxynitrite showed gross structural changes as revealed by physico–chemical results. Peroxynitrite–mediated nitration and oxidation appears to have generated highly immunogenic epitopes on H2A histone because rabbits challenged with peroxynitrite–modified H2A produced high titre antibodies. Furthermore, the immunogenicity of peroxynitrite–modified H2A was directly proportional to nitrotyrosine contents. Experimentally produced antibodies against peroxynitrite–modified H2A were polyspecific because they showed specificity for the immunogen as well as cross-reaction with nitrated epitopes of other proteins modified by peroxynitrite. The preferential binding of peroxynitrite–modified histones by autoantibodies derived from SLE and RA sera points out the role of oxidatively modified and nitrated proteins in the initiation/progression of SLE and RA, at least in a sub-group of these patients. Moreover, oxidatively modified and nitrated protein antigens, rather than nucleic acid antigens, appear to be more suitable as trigger for these disease because of abundance of tyrosine rich proteins in circulation as well as in synovial membrane.

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